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University  
of Glasgow

## Optimisation of Analytical Methods for the Detection of Cannabinoids and Nicotine in Hair

Thesis Submitted in Accordance with the Requirements of the University of  
Glasgow for the Degree of Doctor of Philosophy

By

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August 2016

## Abstract

Unlike conventional biological samples (blood and urine), hair samples have a much wider detection period and can provide a retrospective timeline of an individual's drug use. However, the most crucial issue facing hair analysis is the avoidance of false-positive results caused by passive exposure to the drug. Passive exposure could be a result of direct contact with the consumed material or its smoke. This issue is of great concern especially with the drugs that have a greater potential for external contamination. Common examples of these are cannabis and nicotine, two drugs that are by far the most used drugs worldwide.

The work presented in this thesis describes the development and validation of three analytical methods for cannabis and nicotine in hair matrices. These methods were then employed to analyse authentic hair samples and their washes.

The first method involved liquid-liquid extraction (LLE) of the cannabinoids,  $\Delta^9$ -tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN) and metabolite 11-hydroxy- $\Delta^9$ -tetrahydrocannabinol (11-OH-THC) from hair followed by analysis using standard gas chromatography-mass spectrometry (GC-MS). Cyclohexane: EtOAc (3/1, v/v) was found to be the best extracting solvent for THC, CBD, CBN and 11-OH-THC. The percentage of extraction recovery for all four analytes ranged from 87.9% to 97.2%.

The second method involved solid-phase extraction (SPE) of the main metabolite 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) from hair followed by analysis using two-dimensional gas chromatography-mass spectrometry (2D GC-MS). The SPE method provided a clean extract with an acceptable extraction recovery (approximately 50%).

Authentic hair samples were then collected from 20 known cannabis users admitted to Al-Amal addiction hospital in Jeddah, Saudi Arabia. Cannabis users were interviewed at the time of sample collection and self-reported their cannabis use history. Concentrations of different cannabinoids were then measured using the validated methods. The aim of this project was to investigate the potential value of measuring cannabinoid concentrations in hair. The detected concentrations ranged from 0.11 to 0.34 ng/mg for THC, 0.2 to 4.42 ng/mg for

CBD, 0.31 to 1.02 for CBN, and 2.14 to 7.01 pg/mg for THC-COOH. Surprisingly, THC has a very low detection rate, whereas, CBD and THC-COOH had the highest detection rate of all cannabinoids. The relationship between measured concentrations and use history was then subject to statistical analysis. There was no significant correlation found between concentrations of cannabinoids in hair and the use history.

The third method involved methanolic extraction of nicotine and cotinine from pet dogs' fur followed by analysis by zwitterionic hydrophilic interaction liquid chromatography tandem mass spectrometry (ZICHILIC-MSMS). Further clean-up of the fur methanolic extract was found to be problematic. Centrifugation and direct analysis was found to be the best approach. The tandem MS allowed for low detection limits. The aim of this project was to investigate the association between dog fur nicotine and cotinine concentrations and owner-reported exposure to environmental tobacco smoke. 66 fur samples were collected from 41 dogs at two time points. Total nicotine and total cotinine were quantified in unwashed fur samples using the validated method. Statistical analysis revealed a significant difference in the mean concentrations of nicotine and cotinine in different exposure groups. By providing information on dog's exposure to environmental tobacco smoke (ETS) over time, fur analysis may be useful in assessing dogs and companion owner's histories of exposure to ETS.



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## Acknowledgement

I am deeply grateful to my principal supervisor, Dr. Gail Cooper, for her immense support, continuous encouragement and motivation throughout my PhD studies. My thanks also extend to my secondary supervisor Dr Fiona Wylie for her invaluable guidance, particularly for giving her time to advise on the ethical approval application and write up of the thesis.

A big thank you to staff and students at Forensic Medicine and Science, University of Glasgow, for all their help, particularly Denise McKeown and Iain MacDonald. for their technical support on the instruments.

I would like to thank Dr Ahmed Alasmari for providing the authentic hair samples and self-report data from cannabis users, and Miss Natalie Hutchinson for providing the pet dog's fur samples and owner-report data on their tobacco smoke exposure

I would like to thank Roaa Baghdadi from research department in Saudi ministry of health for her great help on ethical approval application process.

I wish to offer my sincere gratitude to my parents for their emotional support and prayers. I am also grateful to my wife Nourah, and my children Rakan, Ahmed, and Rudeena for their patience, support and love. I also wish to thank all my friends, especially Dr. Jahad AlGhamdi and Dr. Abdulaziz Alqadi for their support during my stay in Glasgow.

Finally, I would like to thank Saudi Ministry of Interior for providing financial assistance during the early years of this work towards completing my PhD degree.

## **Author's declaration**

"I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution".

Signature \_\_\_\_\_

Printed name \_\_\_\_\_

## Abbreviations

2D	Two dimensional
%CV	relative standard deviation
%PAR	% peak area ratio
$\Delta$ 8-THC	$\Delta$ 8-tetrahydrocannabinol
$\Delta$ 9-THC	$\Delta$ 9-tetrahydrocannabinol
11-OH-THC	11-hydroxy- $\Delta$ 9-tetrahydrocannabinol
2D GC-MS	Two-dimensional gas chromatography mass spectrometry
6-MAM	6-monoacetylmorphine
AAFS	American Academy of Forensic Scientists
Ab-Ag	Antibody-antigen
amu	Atomic mass units
ANRF	American Nonsmokers Right Foundation
ACN	Acetonitrile
Bk Inl Temp	Cryotrap
BSTFA	N,O-bis-(trimethylsilyl)-trifluoroacetamide
CB1	Central cannabinoid receptors
CB2	Peripheral cannabinoid receptors
CBC	Cannabichromene
CBD	Cannabidiol
CBDA	Cannabidioloic acid
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CBN	Cannabinol
CEDIA	Cloned enzyme donor immunoassay
CFT	Capillary Flow Technology
CI	Chemical ionisation
CMC	Cell membrane complex
CNS	Central nervous system
COC	Cocaine
CYP	Cytochrome P450
<i>d</i>	Deuterated compound
DCM	Dichloromethane
dH <sub>2</sub> O	Deionised water
EC	Electron capture
ECD	Electrochemical detection
EI	Electron impact mode
ELISA	Enzyme-linked immunosorbent assay
EMIT	Enzyme-multiplied immunoassay technique

EPA	Environmental Protection Agency
EtOAc	Ethyl acetate
ETS	Environmental tobacco smoke
EWDTs	European Workplace Drug Testing Society
FCTC	Framework Convention on Tobacco Control
FID	Flame ionisation detector
FMS	Forensic Medicine and Science laboratory
FN	False-negative
FP	False-positive
FPIA	Fluorescence polarization immunoassay
FS	Full-scan
FTC	Forensic Toxicology Council
GC	Gas chromatograph
GC-GC	Comprehensive 2D gas chromatography
GC-MS	Gas chromatography mass spectrometry
GTFCh	German Society of Toxicological and Forensic Chemistry
HCl	Hydrochloric acid
HFBA	Heptafluorobutyric anhydride
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol (derivative)
HF	Hair follicle
HILIC	Hydrophilic interaction chromatography
HNC	Hair nicotine concentration
HPLC	High Performance Liquid Chromatography
ISTD	Internal standard
IARC	International Agency for Research on Cancer
ICR	Incorporation rate isopropanol
ID	Internal Diameter
IF	Intermediate filament
IS	Involuntary smoking
KAPs	Keratin associated proteins
KIFs	Keratin intermediate filaments
KIMS	kinetic interaction of microparticles in solution
KOH	Potassium hydroxide
KSA	Kingdom of Saudi Arabia
LC-MS	Liquid chromatography mass spectrometry
LLE	Liquid liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
LR-	Negative likelihood ratio
LR+	Positive ikelihood ratio

m/z	Mass to charge ratio
MDGC	Multidimensional gas chromatography
MeOH	Methanol
MOH	Ministry of Health
MRM	Multiple reactions monitoring
MS	Mainstream smoke
MS <sub>bg</sub>	Mean square between groups
MSD	Mass selective detector
MSMS	Tandem mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
MS <sub>wg</sub>	Mean square within groups
MTBE	Methyl tert-butyl ether
MVLS	Medical, Veterinary and Life Sciences
N <sub>2</sub>	Nitrogen
NaOH	Sodium hydroxide
NCI	Negative chemical ionisation
ND	Not detected
NIST	National Institute of Standards
NNO	Nicotine-N'-oxide
NPV	Negative predictive values
NRC	National Research Council
NSD	Nitrogen-selective detection
OCI	Positive chemical ionisation
PAHs	Polycyclic aromatic hydrocarbons
PCM	Pressure Control Module
PFPA	Pentafluoropropionic anhydride
PFPOH	2,2,3,3,3-Pentafluoro-1-propanol
PM <sub>2.5</sub>	Fine fraction of particulate matter
PPV	Positive predictive values
PS	Passive smoking
QC	Quality control standard
RBCs	Red blood cells
RF	Radio frequency
RIA	Radioimmunoassay
RT	Retention time
S/N	Signal to noise ratio
SALL	Supported-assisted liquid-liquid extraction
SAMHSA	Substance Abuse and Mental Health Services Administration
SHS	Second-hand smoke
SIM	Selected ion monitoring



SOFT	Society of Forensic Toxicologists
SoHT	Society of Hair Testing
SPDE	Solid-phase dynamic extraction
SPE	Solid phase extraction
SPME	Solid-phase microextraction
SQ	Tingle quadrupole
SS	Tidestream smoke
SWGTOX	Scientific Working Group for Forensic Toxicology
TFAA	Trifluoroacetic anhydride (derivative)
TFAA	Trifluoroacetic anhydride
THC	$\Delta^9$ -tetrahydrocannabinol
THCA-A	Tetrahydrocannabinol acid A
THC-COOH	11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid
THC-COOH-glu	11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid glucuronide
TIC	Total ion chromatogram
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl (derivative)
TN	True-negative
TP	True-positive
TSNAs	Tobacco-specific nitrosamines
UK	United Kingdom
UKIAFT	United Kingdom and Ireland Association of Forensic Toxicologists
US	United States
USA	United States of America
UV	Ultraviolet
$V_d$	Volume of distribution
WHO	World Health Organisation
WS	Working solution mixture
ZIC-HILIC	Zwitterionic hydrophilic interaction chromatography

# Chapter 1 Introduction

## 1.1 Forensic toxicology

**‘ALL SUBSTANCES ARE POISONS. THERE IS NONE WHICH IS NOT A POISON. THE RIGHT DOSE DIFFERENTIATES A POISON AND A REMEDY’**

Paracelsus: 1493 - 1541

The above quote was given to us in the sixteenth century by whom is nowadays known as the father of toxicology, Paracelsus. This quotation, which is sometimes condensed to “the dose makes the poison”, is often referred to as classic toxicology maxim. The word ‘toxin’, in toxicology, is derived from ancient Greek ‘τοξικόν toxikon’. According to Dorland's Medical Dictionary, a toxin is a poisonous substance produced within living cells or organisms, while toxicology is the science or study of poisons. The above definition of toxins excludes synthetic toxins, such as some drugs of abuse, as they are made by artificial processes. A poison, according to the same dictionary, is defined as a substance that cause disturbances in organisms, usually by chemical reaction or other activity on the molecular scale, when an organism absorbs a sufficient quantity(1). Therefore, we can say that all toxins are poisons but not all poisons are toxins. Toxicology can be broken down into different disciplines based on the purpose of the measurement of a toxin and/or the toxicity and source of sample. These disciplines deal primarily with analytical chemistry, bioassay, and applied mathematics.

The five main toxicology disciplines are molecular, veterinary, environmental, clinical and forensic toxicology. Molecular Toxicology is the study of the effects of various chemical components on living organisms. Veterinary Toxicology focusses on the diagnosis and treatment of poisoning exclusively in animals. Environmental Toxicology is concerned with the study of chemicals that contaminate food, water, soil, or the atmosphere. Clinical and forensic toxicology are similar branches of toxicology and both are involved in the detection and identification of toxic chemicals and their metabolites in biological samples. The difference is that clinical toxicology is usually hospital-based, and therefore, the analysis request is received exclusively, from hospital physicians, while forensic

toxicology is often carried out for administrative and/or medico-legal purposes and has a wider range of customers including but not limited to; police officers, court employees, and defendants.

Forensic toxicology has different applications and hence can be further classified into different sub-categories. The Forensic Toxicology Council (FTC) was formed in 2009 in the United States to represent the interests of the major professional organisations, to provide information to government and policy makers regarding the field, and promote the advancement and development of forensic toxicology. FTC classified forensic toxicology into four main disciplines; namely (1) Death Investigation (or post-mortem) Toxicology, (2) Human Performance Toxicology, (3) Doping Control and (4) Workplace Drug Testing (2). Death investigation toxicology is typically required to confirm or eliminate the possibility of a drug overdose and its potential contribution to death. Human performance toxicology deals with the effect of alcohol and drugs on human performance and behaviour, and the potential medico-legal consequences of drug and alcohol use. This category includes investigation of impaired driving and drug facilitated crime (DFC) which may require determination of possible exposure to a substance hour to weeks after an alleged offence. Doping control is a well-established activity in sport and is often required to monitor athletes for the use of list of prohibited substances both in and out of competition. Workplace drug testing is carried out usually, but not exclusively, in industries and professions of safety nature and security critical, such as transportation and the armed forces.

Before toxicology testing can go forward, suitable samples need to be collected. Urine and blood or its component parts, that is, plasma or serum, are the most commonly employed matrices in forensic toxicology. Alternative matrices include hair, saliva and oral fluid, nail, meconium, sweat, amniotic fluid, breast milk, vitreous humour, bone and bone marrow, liver and brain (3). Most of the biological samples are complementary and each sample type has different advantages. The selection of the sample type is hugely influenced and dictated by the purpose of the analysis, the targeted substances and the time of sample collection. For instance, the typical matrix for workplace or court-ordered drug testing is urine. Blood is preferred and most frequently collected when the behaviour of an individual is to be the subject to an investigation. Interpretation of impairment

from drug levels in urine is not possible. Oral fluid is an excellent matrix to show recent drug use, however, it contains only the parent drug substance rather than drug metabolites for some drugs. For example, there is almost no carboxy metabolite of THC present in oral fluid (4). Conversely, hair is the best option to monitor long-term exposure or use but is not as suitable to assess recent exposure. Hair as a biological matrix for drug detection has increasingly received attention in recent years, and is utilised in the work presented in this thesis. The physiological and anatomical properties of hair, the potential and benefits of hair as a matrix for drug detection, as well as its associated limitations and challenges, will be discussed in this chapter.

As mentioned earlier, results of forensic toxicology testing may be used for legal purposes and hence in court proceedings. Therefore, the first necessary component of the testing process is to guarantee the validity of test specimens. This is accomplished through chronological documentation of sample status from the time of collection until analysis. This process is called 'chain of custody' (5).

Analyses in forensic toxicology are typically conducted in two phases; screening and confirmation. Screening tests are intended to provide an indication of whether a particular drug or drug class is present and, typically, involve using immunoassay techniques, which are usually designed to targets a class of drugs, such as opiates, or may be a broad-based screening test using more advanced techniques such as gas chromatography mass spectrometry (GC-MS).

Screening assays usually produce either a negative or a presumptive positive result for one or more drugs or drug classes which are then subjected to a confirmation test. According to the SOFT/AAFS Forensic Laboratory Guidelines, the initial detection of drugs should be confirmed whenever possible by a second technique based on a different chemical principle (5). Typically, a mass spectra based technique is employed for confirmation.

The type of sample, the chemical properties of targeted substances and the required sensitivity could dictate the selection of the appropriate analytical method either during screening or confirmation. The scope of analysis and list of targeted substances varies from one laboratory to another, and is usually determined based on many factors including, but not limited to, the purpose of

the analysis, the prevalence of a substance and availability of the required resources and instrumentation.

## 1.2 Hair

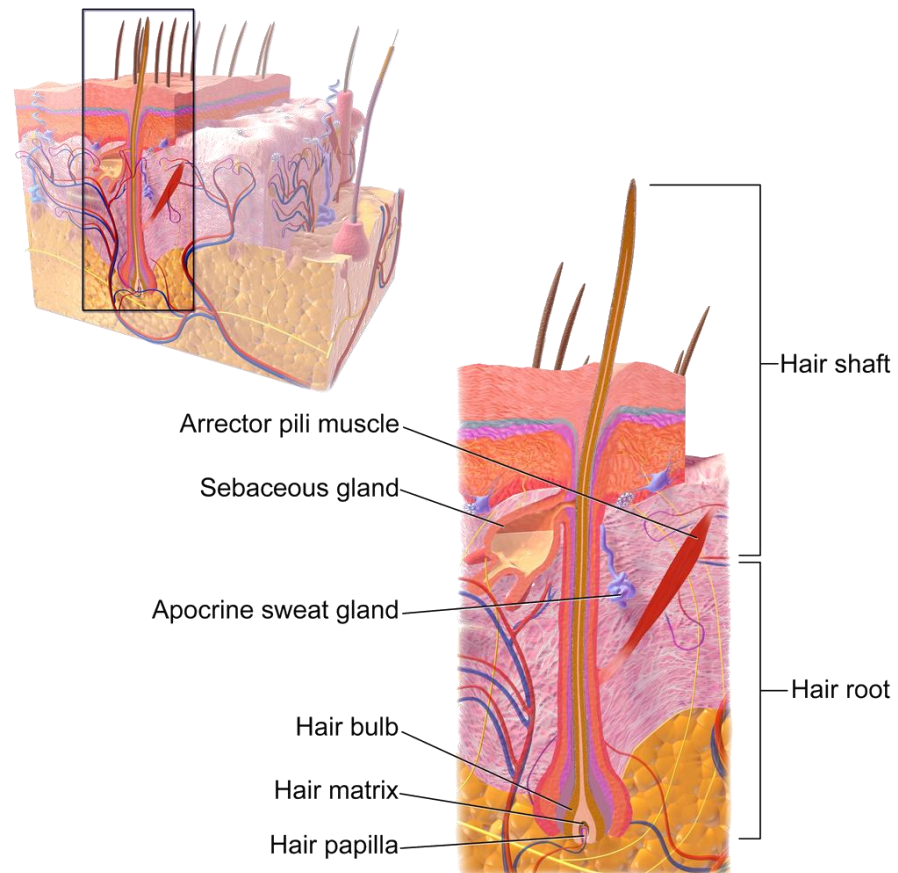
Hair is a fine thread-like strand growing from underneath the skin with a root covering the mammals body. It is commonly dismissed as being of negligible importance, however, the hair follicle (HF) is, in fact, one of human biology's most interesting structures (6). Hair is a mammalian quality that provides numerous important roles. These include; thermal insulation, social and sexual communication, sensory perception (e.g. whiskers), and protection against trauma, noxious insults, insects, etc. These features have very obvious benefits in animals, however it is not yet clear how these may have proved crucial for human survival (7). The primary difference between hair and fur is the word usage. The hair of non-human mammals is referred to as "fur," while humans have hair. So, basically, hair is a characteristic of all mammals. Fur is a reference to the hair of animals.

### 1.2.1 Basic structure of mammalian hair

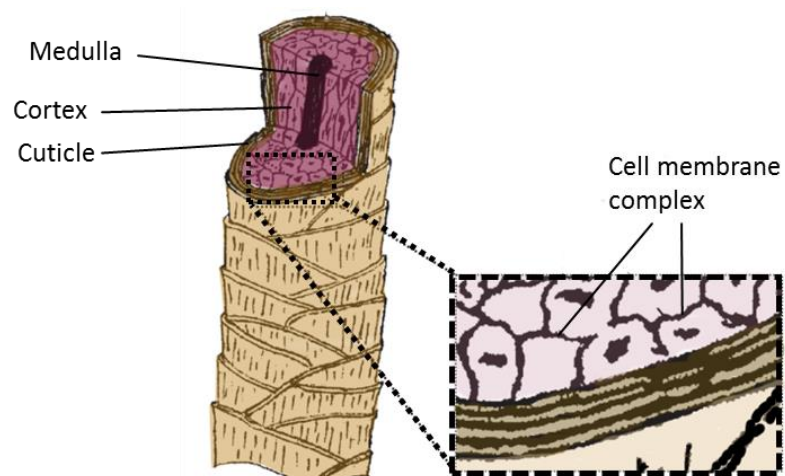
Hair and fur are chemically indistinguishable, and have the same chemical composition (8). Hair consists of a shaft that protrudes above the skin and a root sunk in a pit called the follicle as shown in Figure 1-1. The hair shaft is composed of protein, lipids and contains other trace materials, and consists of three distinct morphological units, moving inwards from the outside of the fibre these are; the cuticle, the cortex and the medulla as shown in Figure 1-2. These three units and accompanying root sheath are formed by the metabolically active dividing cells above and around the papilla of the follicle.

The cuticle is formed of flat overlapping cells separated by the cell membrane complex (CMC). It covers the hair from the root to the tip of the epidermis and acts as a sheath to the hair, protecting it from the environment (9,10). The cortex, or shell of the hair shaft, surrounding the medulla, is composed of elongated, fusiform cells (spindle-shaped), keratinized filaments aligned parallel to the length of the hair as shown in Figure 1-3. It is the main component in hair

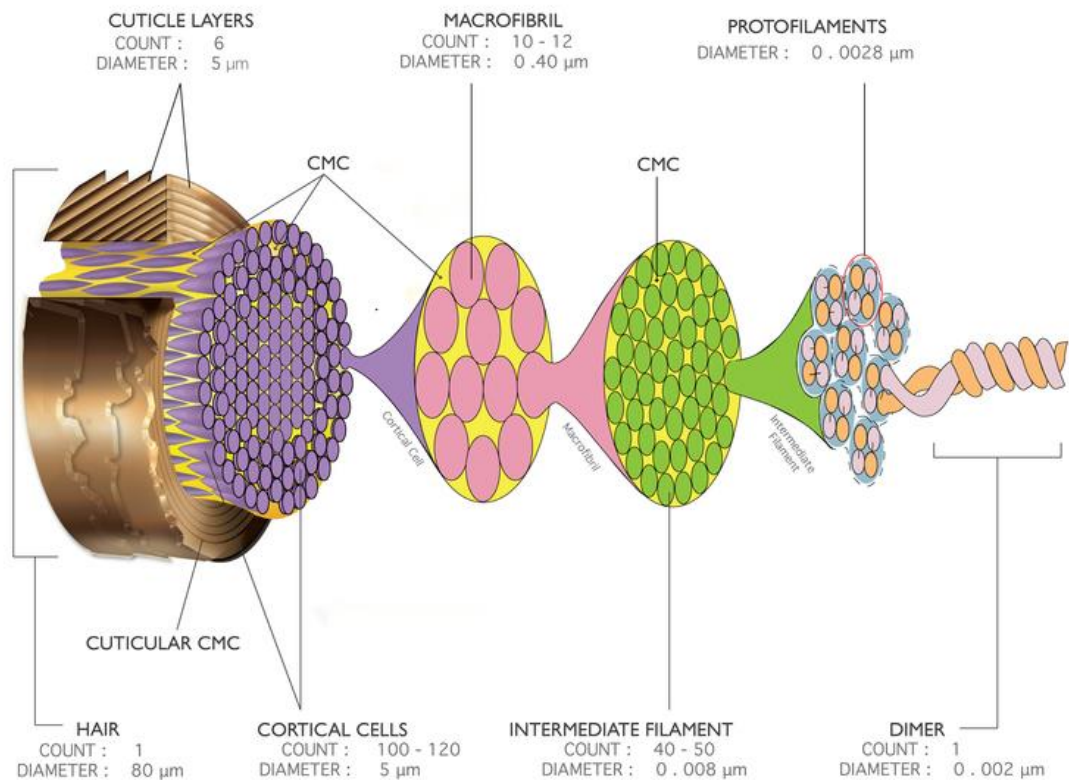
and represents an average of 90% of total hair mass (11). The cortex may contain acortical fusi, pigment granules, and/or ovoid bodies.



**Figure 1-1 Hair follicle (root) and hair shaft (12).**

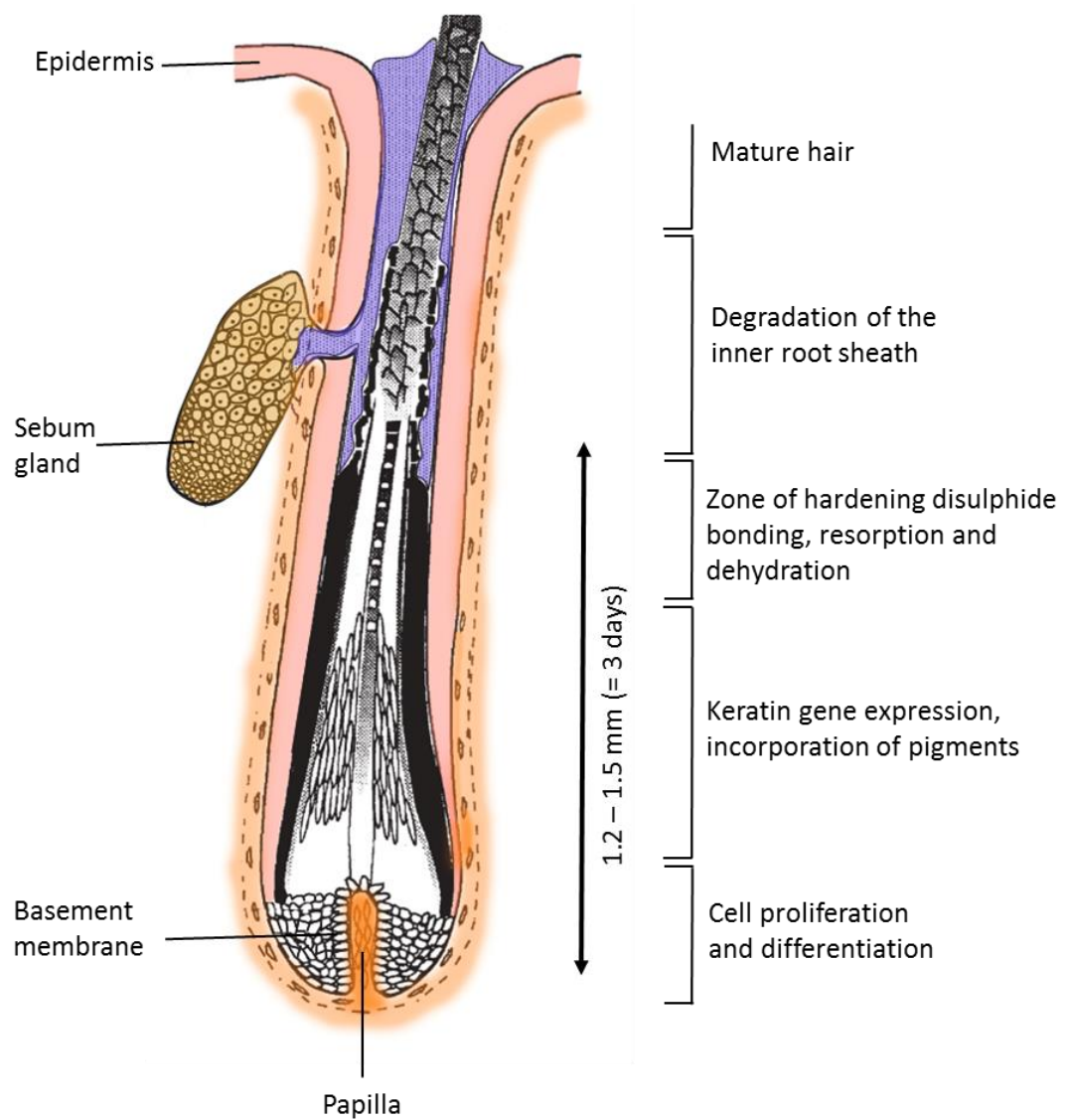


**Figure 1-2 Structure and constituents of the human hair shaft. Adapted with permission from ref (13)**



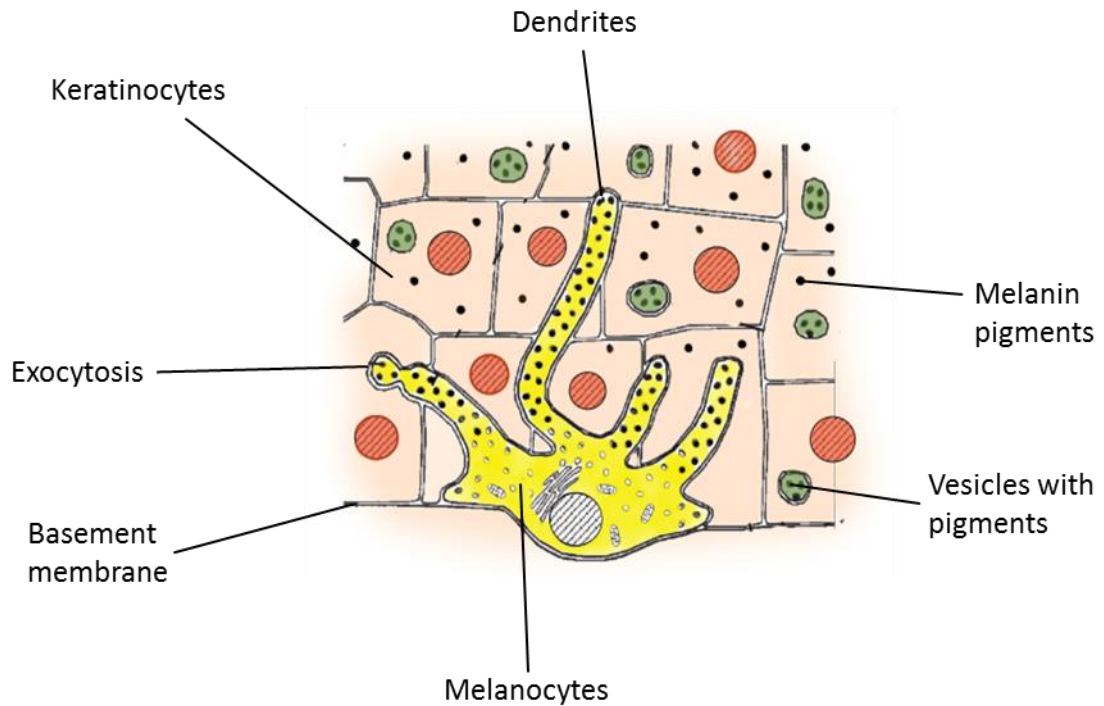
**Figure 1-3 Structure and constituents of the hair shaft cortex. from ref (14) with permission.**

The medulla is a group of high porosity cells in the central part of the hair, located usually in the large terminal hair fibres. It should be noted that the medulla is sometimes absent in human hair (15). Studies on incidence of medullation in human hair revealed a considerably lower presence of medulla in scalp hair in comparison with axillary and pubic hair (10). The medulla's role is still not well understood and little research has been conducted on its chemistry and other properties (14). The root of a single hair is situated in an epidermal tube known as the hair follicle, sunken into either the dermis or the subcutaneous tissue. The components of the follicle are shown in Figure 1-4. It is surrounded by a rich blood capillary system that delivers necessary nutrients, elements and metabolic material to the growing hair (16). The germination centre around the hair bulb papilla is formed by keratinocytes and melanocytes present on the basement membrane, shown in Figure 1-5.



**Figure 1-4 Formation of hair in a follicle from matrix cells on the basement membrane to the mature hair shaft. Adapted with permission from (13)**





**Figure 1-5 Melanocyte on the basement membrane of the cortex. Adapted with permission from (13)**

Melanin is synthesised in melanocytes and released into the keratinocytes in vesicles (melanosomes) by an exocytotic mechanism. In the keratinocytes, the membranes of the vesicles are digested and melanin pigments remain. Three glands, the sebaceous gland, the apocrine gland, and the eccrine (sweat) gland, are closely associated with hair follicles (17). Typically, the hair follicle and the sebaceous gland are merged both anatomically and functionally to form a pilosebaceous unit. The ducts of apocrine and sebaceous glands, only, empty their secretions into the hair follicle, while the eccrine glands do not (17). Sebaceous glands and eccrine glands are distributed nearly over the entire surface of the body, on the other hand, apocrine glands are present in the axilla, the ear canal, the eyelids, and the perineal region. Sebum is the name for sebaceous gland secretion and is reported to contain mainly triglycerides (41%), free fatty acids (16%), wax esters (25%) and squalene (12%) (11). Sebaceous glands and sweat glands are one of the proposed routes in which drugs and their metabolites are incorporated into hair.

### 1.2.2 Chemical composition

Human hair composition is influenced by its moisture content (up to 32% by weight) (18). It consists of approximately 65% to 95% proteins and other constituents such as water, lipids, pigment, and trace elements. Proteins are condensation polymers of amino acids. There are two groups of proteins that comprise a hair fibre; keratin and keratin associated proteins (KAPs). The keratin present in hair is helical and constructed into filaments and keratin intermediate filaments (KIFs) which are responsible for the fibrous structure of the hair. KIFs are part of a bigger family of molecules; the intermediate filament family (IF). IFs are measured to be 8-12 nm in diameter; KIFs are roughly 9 nm in diameter (19). Lipids are found within the hair fibre, some of which are in crystalline form, external lipids excreted from the sebaceous glands are found in the cuticle. Some trace elements such as the heavy metals, iron and lead, are also found in hair.

### 1.2.3 Hair growth cycle

Hair growth starts in cells around the papilla in a germination centre. These cells are a collection of epithelial cells called the matrix cells and are responsible for the growth of hair. The matrix cell cycle is one of the most rapid of all human tissues. As a result of matrix cells mitosis, they become larger and move up the follicle into the keratogenous zone. Here the cells synthesise melanin pigment and begin to 'keratinize'. Long fibers are formed through the cross-linking of the sulfhydryl groups in amino acids like cysteine. Gradually, the hair cells die and decompose by eliminating the cell nucleus and releasing water. Humans, unlike most other mammals, grow their hair in a mosaic pattern with noteworthy independence of growth and pigmentation resides in the individual hair follicle. Other mammals grow their hair synchronously or as a wave. The hair growth cycle describes the changing histological morphology of the shaft and of the follicle over time. Starting with the anagen phase, the follicle and its shaft progress through the catagen phase, and finally the telogen phase as shown in Figure 1-6. Each strand of hair on the human body is at its own stage of development. Once the cycle is complete, it restarts and a new strand of hair begins to form. In humans, the anagen phase lasts for three to five years, the catagen phase for a few weeks and the telogen phase for two months (20). The anagen phase is known as the growth phase. The longer the hair stays in the anagen phase, the longer it will

grow. About 85% of the hairs are in the anagen phase at any given time. It is thought that drugs and trace elements are incorporated into hair at this time of intense metabolic activity (17). The next phase is **catagen** which is also known as the transitional phase. The first sign of catagen phase is the cessation of melanin production in the hair bulb and apoptosis of follicular melanocytes (21). During this time, the hair follicle shrinks due to disintegration and the papilla detaches and cuts the hair strand off from its feeding blood supply. Ultimately, the follicle is a sixth of its original length, causing the hair shaft to be pushed upward causing an increase in length of the terminal fibres. During the **telogen** or resting phase the follicle remains dormant. Only 10% to 15% of the hairs are in this phase of growth at any given time. In this phase the epidermal cells lining the follicle channel continue to grow as normal and may accumulate around the base of the hair, temporarily anchoring it in place and preserving the hair for its natural purpose without demanding the body's resources needed during the growth phase. At some point, the follicle will begin to grow again, softening the anchor point of the shaft initially. The hair base will break free from the root and the hair will be shed. Within two weeks the new hair shaft will begin to emerge once the telogen phase is complete.

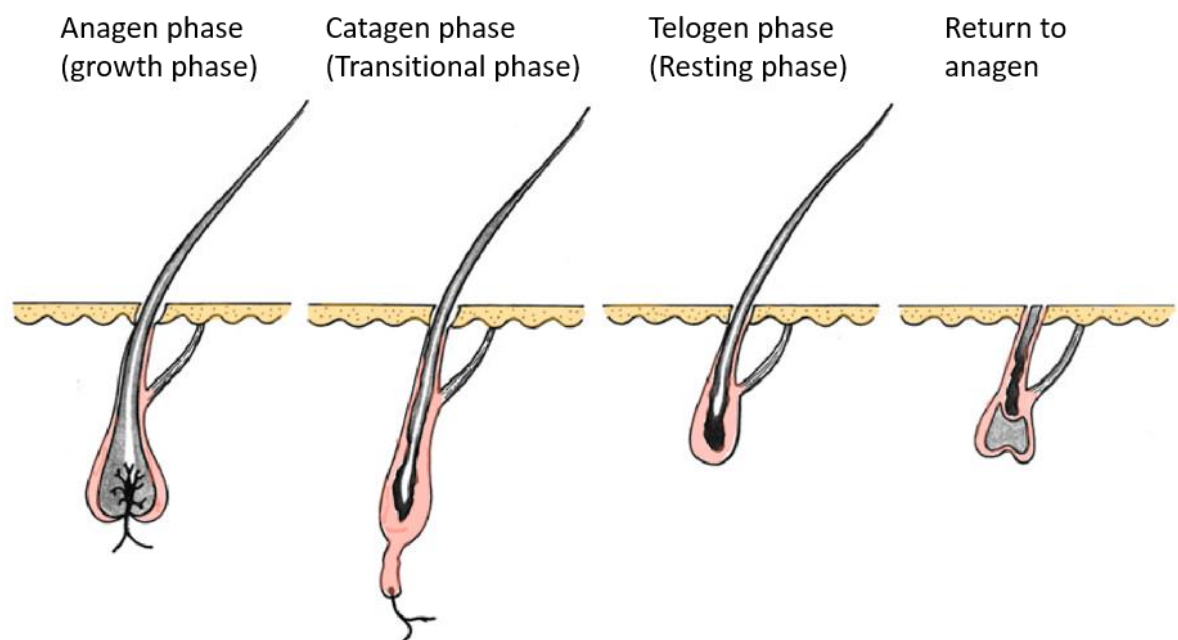


Figure 1-6 Different phases of the hair cycle. Adapted with permission from (10)

### 1.2.4 Rate of hair growth

The duration of growth cycles is harmonized by many hormones and cytokines and influenced by many factors including; ethnicity, gender, the body site where the hair is growing, age, stage of development, dietary habits, environmental alterations like day-length, disease, or cosmetic use (22,23). There is little evidence to suggest that this rate can be increased by external factors such as grooming or shaving (6). Hair in the vertex region of the scalp is often selected as a test specimen because it has approximately 85% of hair follicles in the anagen phase (least variation) and has the fastest growth rate. Different studies were carried out to estimate growth rate of human scalp hair. Harkey (17) reported an average growth rate of 0.44 mm/day. Pötsch *et al.* found a variation between 0.07 and 0.78 mm/day, with 82% of the examined population between 0.32 and 0.46 mm/day (24). For the purpose of interpretation, one centimetre per month (1 cm/month) is generally accepted in the scientific community as growth rate of scalp hair for an adult male or female (25,26). Therefore, a short length of hair (2-3 cm) from the scalp can theoretically represent the exposure to drugs over the past two to three months.

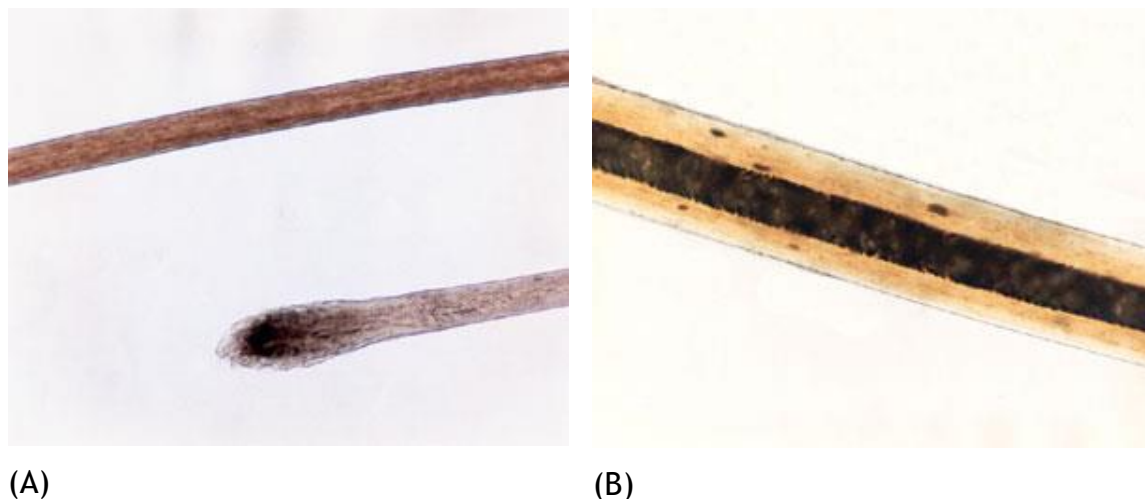
### 1.2.5 Hair pigmentation

Melanins in mammals are formed in specialised cells called melanocytes, which enclose distinct cytoplasmic organelles known as melanosomes. The process of formation of melanin is called follicular melanogenesis and takes place in the hair bulb. The active melanocytes, which exist in the anagen hair follicle, transfer melanin mainly to the hair shaft cortex, to a lesser extent to the medulla, and only rarely to the hair cuticle (27). Hair color is genetically controlled and is among the most diverse of the pigmentation phenotypes. In the past, it was proposed that the variations in the proportion of two, chemically, different forms of melanin pigment, created from the same melanocyte, would determine the human hair and skin colour. Eumelanins are believed to be responsible for dark hair, while pheomelanins for yellow-to-red colours (28). In 2000, Prota criticised these traditional concept and proposed a four-class system for defining hair color (29). According to Prota's system, the diversity in hair colour is caused by four types of melanin. In addition to the known melanin pigments eumelanins and pheomelanins; their oxidative products oxyeumelanin and oxypheomelanin are

believed to have a role to play in determining hair colour. In Prota's proposal, presence of the intact eumelanin pigment will result in black to dark brown hair, on the other hand, hair that contains more of an oxidative breakdown product of eumelanin, namely oxyeumelanin, tend to be lighter. Presence of large amounts of oxyeumelanin will result in blond hair.

### **1.2.6 Human scalp hair and dog's fur**

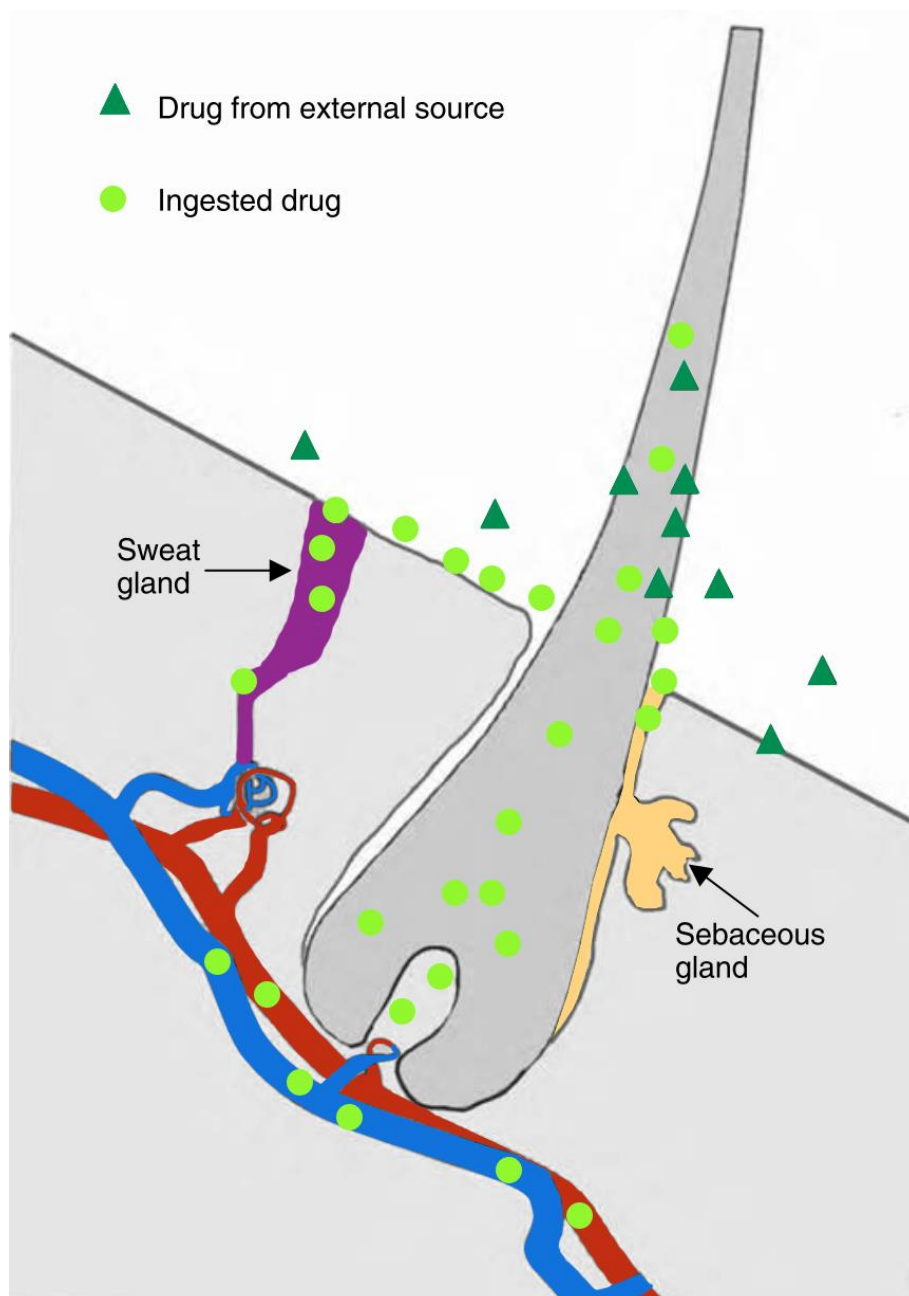
Human hairs are divided into three basic types: vellus, intermediate and terminal hair. These hair types are distinguishable based on different characteristics including; length, colour, texture, shape and diameter and, also, based on their region of growth. The vellus hair is the very small colourless hair that covers almost the whole human body, clearly seen on the bald scalp or forehead. The intermediate hair is that found on the hands and legs of adults that has an intermediate size and length and intermediate cross sectional area. The terminal hair is usually coloured, long hair and has a wide cross sectional area which is mostly found in the scalp, beard, moustache, eyelashes, eyebrows, pubic hair and axilla. The main focus of this research is mostly on the terminal scalp hair as it is the chosen specimen for analysis. A human hair fibre is approximately 50 - 100  $\mu\text{m}$  in diameter and can grow up to metres in length (30). The average human displays an estimated total number of 5 million hair follicles, of which 80,000-150,000 are located on the scalp (31). Domestic animals, such as pet dogs, are distinguished primarily through their medullary structure and pigmentation (32). In human hairs, the medulla is generally amorphous in appearance, whereas in animal hairs, its structure is frequently very regular and well defined. This core, in animals, allows for the coating of the hair to provide excellent thermal insulation, whereas human hair lacks this ability and does nothing to provide temperature regulation for the body. In humans, pigment granules are commonly distributed toward the cuticle as shown in Figure 1-7 (A). Animal hairs have the pigment granules commonly distributed toward the medulla, as shown in Figure 1-7 (B).



**Figure 1-7** Photomicrograph of (A) human hair and (B) dog's fur

### 1.3 Mechanism of drug incorporation

The precise mechanisms involved in the incorporation of drugs into hair has not been clarified completely and more research is still necessary. However, the factors that influence incorporation of drugs into hair have been studied extensively in the literature (24,33-40). Three models have been suggested in the literature to explain incorporation of drugs into hair: drugs can enter the hair through (1) active or passive diffusion from the bloodstream nourishing the dermal papilla, (2) diffusion from sweat and sebaceous glands that release its secretions into the growing or mature hair fibre, or (3) diffusion into the mature hair fibre from smoke, vapours, powders or raw drug materials. A combination of these routes is likely to be the most realistic model to explain the drug profiles in hair (27). A schematic view of three proposed incorporation routes of drugs into hair is shown in Figure 1-8. So far, the contribution of the different routes is not fully understood and may vary greatly between substances and individuals.



**Figure 1-8 Incorporation routes of drugs into the hair.** Reproduced from (27). (© 2007 by Taylor & Francis Group, LLC)

### 1.3.1 Drug properties influencing incorporation

The physiochemical properties of drugs are assumed to play a role in their incorporation into hair, the main ones being lipophilicity, basicity and melanin affinity. Due to the chemical nature of the cell membrane, non-polar, more lipophilic parent drugs are more likely to pass from the bloodstream to the hair forming cells than polar, more hydrophilic drug metabolites (34). Nakahara *et al* reported a positive correlation of 0.770 between lipophilicity and incorporation rate into rat hair for nineteen basic drugs of abuse (39).

Ionisation of drugs at physiological pH has a great impact on their incorporation into hair. Basic drugs exist as cationic molecules at physiological pH and should preferentially bind to melanin in pigmented hair. In a rat model and at physiological pH, Rollins *et al* reported a higher incorporation rate for the weak base, codeine, than the weak acid, phenobarbital (41). Nakahara *et al* came to similar conclusion after examining the incorporation rates of twenty acidic, basic and neutral drugs of abuse using the rat model (39). They observed a 3600-fold difference between the incorporation rates of cocaine (highest rate) and THC-COOH (lowest rate). Borges *et al* reported a better incorporation rate for the amphetamine into both pigmented and non-pigmented hair than its non-basic analogue N-acetylamphetamine (40).

As discussed earlier in section 1.2.5, melanin pigments have been proven to be responsible for hair colour and are thought to be the principal component for binding drugs. Melanin polymers possess many negatively charged carboxylic acid groups in their structure which attract positively charged basic drug molecules under physiological conditions, therefore, melanin affinity is associated with drug basicity. Several researchers have demonstrated that different hair colour and types incorporate different amounts of drugs when exposed under identical conditions. These studies have suggested that coarse, dark hair may incorporate more drug than fine brown or blond hair. Henderson *et al.* studied incorporation of isotopically labelled cocaine (COC) into human hair. Despite the small sample size, the authors reported 2.7 times more COC-*d5* in the non-caucasian group (42). Kronstrand *et al.* examined the concentration of codeine after controlled dose administration versus hair melanin content. The authors reported a linear relationship between codeine concentrations and total melanin ( $r^2=0.86$ ) and eumelanin ( $r^2=0.90$ ) and suggested normalizing concentrations for melanin content (43). Rollins *et al* published their findings for a similar study confirming Kronstrand's conclusion (34). These findings contrast with more neutral and acidic drugs such as THC-COOH and N-acetylamphetamine where a lack of correlation between concentrations of these drugs in hair to hair melanin content has been reported (39,40).



## 1.4 Hair analysis

### 1.4.1 Chronology

In 1858, the first case of the determination of poison in human hair was published in the 'Practical Guide to Legal Medicine' by Hoppe (44). Hoppe succeeded in determination of arsenic in the hair of a body exhumed after 11 years. Almost a century later, in 1954 Goldblum determined barbiturates in the hair of a guinea-pig (45). This was followed in the next two decades by metals and trace element analysis in hair (46-48). At that time, hair was proposed as a promising specimen for assessing dietary status of people. However, Sorenson *et al* soon doubted its validity due to the difficulties in establishing normal ranges due to the variations among laboratories and potentials of external contamination from cosmetic products, air or water (49). In 1979, detection of drugs of abuse in hair was initiated in an important publication by Baumgartner (50). He had investigated opiate addiction by extracting opiates from hair using methanol and 2 hours heating. A number of studies followed during the eighties on detection of other drugs of abuse. One of the keystone papers was published in Germany by Klug in 1980 (44). For the first time, the solid hair matrix was converted into a liquid phase using sodium hydroxide in Klug's work. The common logical expectation at that time was that only what is detected in urine could be seen in hair. This way of thinking continued until the end of the 1980s, when cocaine and benzoylecgonine were quantified and the cocaine concentration was always higher than that of benzoylecgonine. The first determination of cocaine in human hair was carried out by Valente in 1980 (51). Since the nineties, the attention to hair analysis has amplified substantially as a result of the advances in detection technologies offering increased sensitivity. Immunoassays, chromatographic techniques coupled with mass spectrometry (MS) and tandem mass spectrometry (MSMS) (GC-MS, GC-MSMS, LC-MS and LC-MSMS) enabled the detection of even a single dose or presence of drugs in a single hair strand. (27)

### 1.4.2 Pros and cons of hair analysis

Hair analysis for drugs has passed through different stages over the years. It has been sometimes glorified, sometimes condemned, sometimes accepted, sometimes rejected. Knowing its benefits and drawbacks will, certainly, guide concerned authorities when deciding whether or not it is a suitable drug testing method for their purpose. In 1995, the Society of Hair Testing (SoHT) was established to encourage consistency of analytical procedures and results interpretation, and to help to prevent or minimise sample mix-up, wrong sample treatment, analytical errors and wrong interpretation of correct results. Since then, the SoHT has published some recommendations and quality control guidelines about hair sample collection and handling procedures, washing, cut-offs for obtaining positive results, metabolites to be assayed and metabolite-to-parent drug ratios (26,52,53). The main advantages of hair drug testing include; (1) a wide window of detection allowing retrospective detection of exposure to illegal drugs, (2) easy and non-invasive sample collection, (3) easy to store and handle hair samples, (4) difficult to dilute or adulterate hair matrices. Despite the above advantages, hair drug testing has also some drawbacks. The main disadvantages of hair testing for drugs is that; (1) it does not provide information relating to recent drug use (previous 7 to 10 days), (2) the detection period usually relies on the length of the subject's hair, (3) there are still disagreements on many factors that may lead to bias or wrong interpretation of the results, particularly concerning external contamination, cosmetic treatments, ethnic and genetic variation and a lack of understanding of drug incorporation mechanisms. To sum up, hair is a useful alternative to blood or urine and a complementary matrix providing important information in drug-related investigations. Interpretation of hair testing results are sometimes challenging due to many factors that may bias the result and must be considered carefully.

## 1.5 General aims and objectives

From the literature it is obvious that there are many variables that must be considered when interpreting the presence of drugs in hair matrices. Cannabis and nicotine are the most widely used drugs. Despite the fact that they have different physiochemical properties, they both share the same challenge in forensic hair testing due to their presence in smoke and hence the potential for external sample contamination. A better understanding of the analytical procedure and concentrations of these compounds in hair samples collected from human active users (cannabis) or passively exposed companion dogs (nicotine) will greatly aid interpretation. The aims of this study and how they will be achieved are listed below:

1. To optimise an analytical procedure for cannabinoids in human hair and explore the detection rate of different cannabinoids in hair samples collected from Arab users.
  - To develop GC-MS and 2D-GC-MS methods for the determination of THC, CBD, CBN, 11-OH-THC and THC-COOH in hair, which will include the optimisation of sample preparation and extraction procedures.
  - To validate the developed methods in accordance with SWGTOX guidelines.
  - To analyse authentic hair samples collected from Middle Eastern cannabis users admitted for treatment at Al-Amal addiction Hospital, Jeddah, Saudi Arabia.
  - To statistically analyse quantified concentrations against the reported use history.
2. To investigate the value of nicotine and cotinine determination in hair to establish the degree of exposure to environmental tobacco smoke (ETS):
  - To develop and validate an LC-MS-MS method for the determination of nicotine and its metabolites in dog fur.
  - To analyse fur samples collected from dogs exposed to various degrees of ETS.
  - To statistically analyse concentrations of nicotine and cotinine against their exposure parameters.

## 1.6 Outline of thesis

The content of the thesis is outlined as follows. **Chapter 2** provides an introduction to cannabinoids and their pharmacological, physical and chemical properties of different compounds. It also reviews the current and past literature for analysis of cannabinoids in hair matrices. **Chapter 3** discusses the details of the optimisation of extraction methods for the three parent cannabinoids (THC, CBD, and CBN) and the two main metabolites (11-OH-THC and THC-COOH) from hair samples using different techniques including LLE, SPE and SALL. This chapter also reports experiments of different derivatising reagent combinations for identification of THC-COOH in mass spectrometry equipped with a negative chemical ionisation source (NCI). **Chapter 4** describes the two instruments acquisition parameters, principles of keys components in the 2D GC-NCI-MS and validation methods and results for both GC-MS and 2D-GC-MS methods. **Chapter 5** provides the empirical findings of the application the developed and validated methods on authentic hair samples collected from cannabis user. This chapter also studies the relationship and correlation of cannabinoids concentrations with the reported use pattern. **Chapter 6** provides an introduction to environmental tobacco smoke (ETS) and its essential biomarker nicotine and cotinine. It also provides an overview of the pharmacological, physical and chemical properties of nicotine and cotinine, and summary on their analytical methods in hair in the literature. **Chapter 7** reports the method development and validation using LC-MSMS. **Chapter 8** report an application of the validated method on fur specimens collected from dogs exposed to ETS for total nicotine and cotinine quantitation. This chapters also reports a brief explanation of the statistical techniques. Finally, **Chapter 9** provides a brief summary, general discussion, contributions and limitations of the study. In addition, this chapter also includes suggestions for future research.

## Chapter 2 Cannabis

### 2.1 Introduction

The cannabis plant is believed to originate from either Central Asia or near the Altai or the Tian Shan Mountains (54). Although all taxonomists recognize the species *cannabis sativa* (55), the cannabis plant is subdivided by some into three species: *cannabis sativa*, *cannabis indica*, and *cannabis ruderalis* (56) based on typology and morphology of the plants (see Figure 2-1). All three species have no differences in genetics or chemical composition.

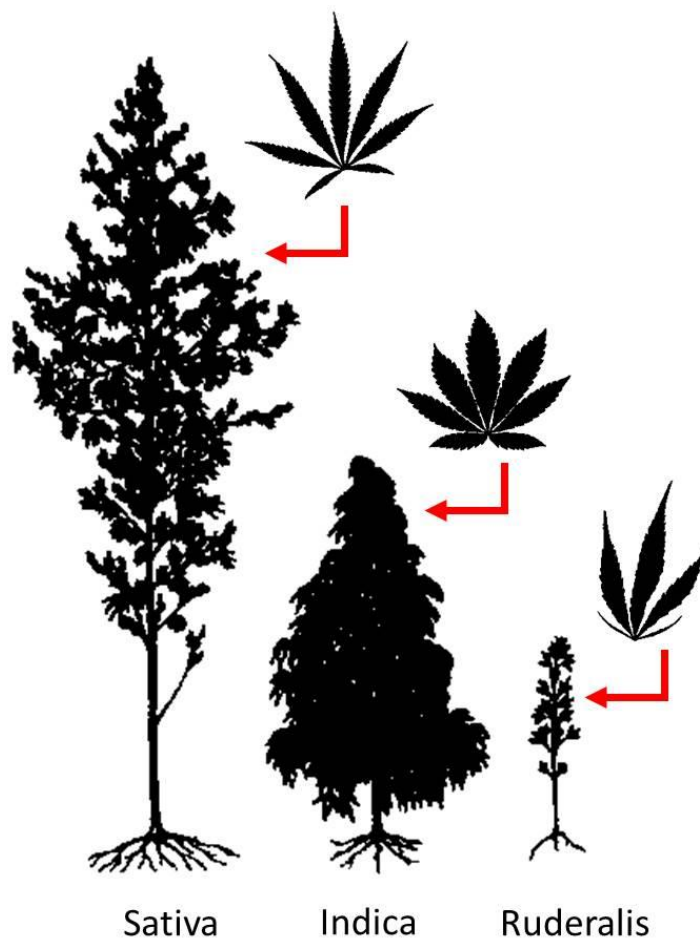


Figure 2-1 Cannabis Species.  
SOURCE: Anonymous / Wikimedia Commons / Public Domain

### 2.1.1 Components of cannabis

The cannabis plant and its products are composed of a wide range of chemicals. Of these, 483 compounds are believed to be present exclusively in cannabis. Yet, approximately 66 cannabinoids have been identified (57). In the past, the term “cannabinoids” was often used to refer to a group of C<sub>21</sub> terpenophenolic compounds that are found exclusively in *cannabis sativa* L (Linnaeus) and which activate cannabinoid receptors. However, recent discoveries of synthetic cannabinoids and the endogenous cannabinoids have caused some confusion over the use of this term. Therefore, cannabinoids can be classified in three main categories according to their origin, endocannabinoids to refer to cannabinoids of human origin, phytocannabinoids refer to cannabinoids of plants origin and synthetic cannabinoids refer to those synthesized in laboratories. In this work, phytocannabinoids are of interest and the term “cannabinoid” will be used throughout to represent this category only. The 66 cannabinoids are divided into 10 subclasses (57). From an analytical point of view, cannabinoids of greater abundance are usually of interest. The main cannabinoids include the primary psychoactive compound  $\Delta^9$ -tetrahydrocannabinol (THC), and the degradative product of the cannabis plant, cannabidiol (CBD), and the oxidation artifact of THC, cannabinol (CBN). From 1980 to 1997, a total of 35,213 samples of seized cannabis products in the United States were analyzed by gas chromatography (GC) (58). The mean THC concentration increased from less than 1.5% in 1980 to 4.2% in 1997. The maximum levels found were 29.9 and 33.1% in marijuana and sinsemilla cannabis, respectively. The main pharmacological characteristics of these compounds and other less abundant cannabinoids are shown in Table 2-1. Of these, the main cannabinoids that are usually targeted in biological samples are THC, CBD, and CBN. CBN was first named a cannabinoid in 1896 by Wood *et al* (59) and its structure was elucidated in 1940 (60). CBD was isolated in 1940 (61), but its correct structure was first explained in 1963 by Mechoulam and Shvo (62). The main active ingredient, THC, was first isolated by Wollner *et al* in 1942 (63), but the correct structure was illustrated 22 years later by Gaoni and Mechoulam in 1964 (64).

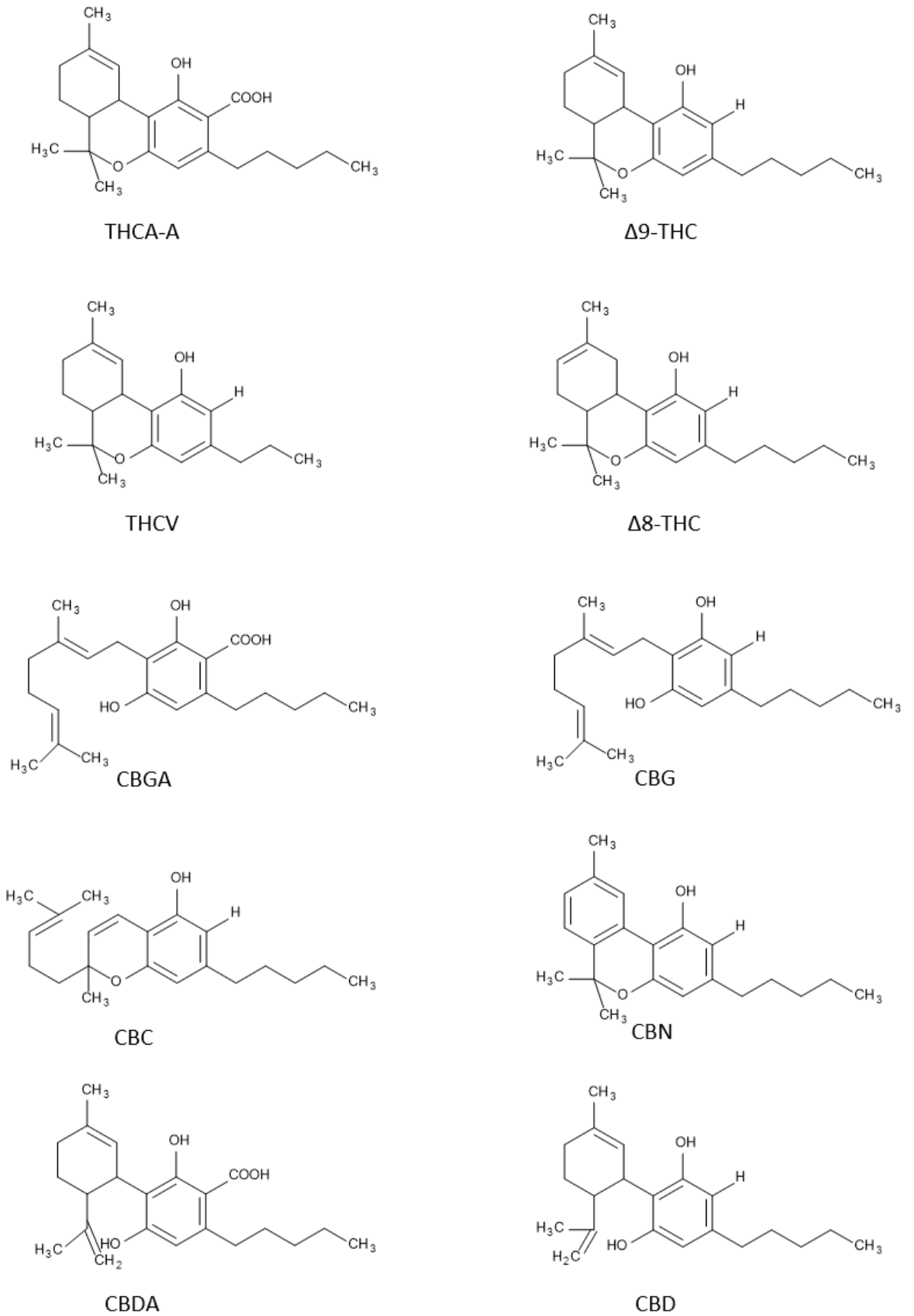
In addition to the above three cannabinoids, tetrahydrocannabinol acid A (THCA-A) is one of the cannabinoids that has recently gained increasing recognition in forensic toxicology laboratories. THCA-A is the non-psychoactive precursor of THC

in cannabis plants. The presence of this cannabinoid exclusively in the cannabis plant may serve as a marker for differentiating between the legal intake of THC medication and external exposure to cannabis products (65). Chemical structures of pharmacologically active cannabinoids and THCA-A are shown in Figure 2-2.

**Table 2-1 Pharmacological actions of the main cannabinoids found in the cannabis plant (57)**

<b>Cannabinoids</b>	<b>Main pharmacological characteristics</b>
<b>Delta-9-tetrahydrocannabinol (<math>\Delta^9</math>-THC)</b>	Primary psychoactive effects
<b>Cannabidiol (CBD)</b>	Anxiolytic, anti-psychotic, analgesic, anti-inflammatory, anti-oxidant, anti-spasmodic
<b>Cannabinol (CBN)</b>	Anti-inflammatory, sedative, antibiotic, anticonvulsant
<b>Delta-8-tetrahydrocannabinol (<math>\Delta^8</math>-THC)</b>	Some psychoactive effects
<b>Cannabigerolic acid (CBGA)</b>	Antibiotic
<b>Cannabigerol (CBG)</b>	Analgesic, anti-inflammatory, antibiotic, antifungal
<b>Cannabichromene (CBC)</b>	Analgesic, anti-inflammatory, antibiotic, antifungal
<b>Cannabidioloic acid (CBDA)</b>	Antibiotic
<b>Delta-9-tetrahydrocannabivarin (<math>\Delta^9</math>-THCV)</b>	Euphoriant, analgesic

Although cannabis plants have similar composition, they can be cultivated to provide different strains. The main cannabinoid types that are usually detected in all strains are THC, CBD, CBN, CBG and CBC. However, there can be significant variation in their quantitative ratios (66). The traditional cannabis gene pools originate either from cannabis sativa, which comprises the vast majority of naturally occurring hemp and drug land races, or from cannabis indica (AKA Cannabis afghanica) from Afghanistan and Pakistan, and has become a component in many modern drug cultivars. The majority of the varieties from these regions, shown in Figure 2-3, are high in psychoactive THC with a widely varying CBD content.



**Figure 2-2 Chemical structure of cannabinoids.**



$\Delta^9$ -tetrahydrocannabinol acid A ( $\Delta^9$ -THCA-A),  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC),  $\Delta^9$ -tetrahydrocannabivarin (THCV),  $\Delta^8$ -tetrahydrocannabinol ( $\Delta^8$ -THC), Cannabigerolic acid (CBGA) cannabigerol (CBG), cannabichromene (CBC), cannabinol (CBN), cannabidioloic acid (CBDA) and cannabidiol (CBD).

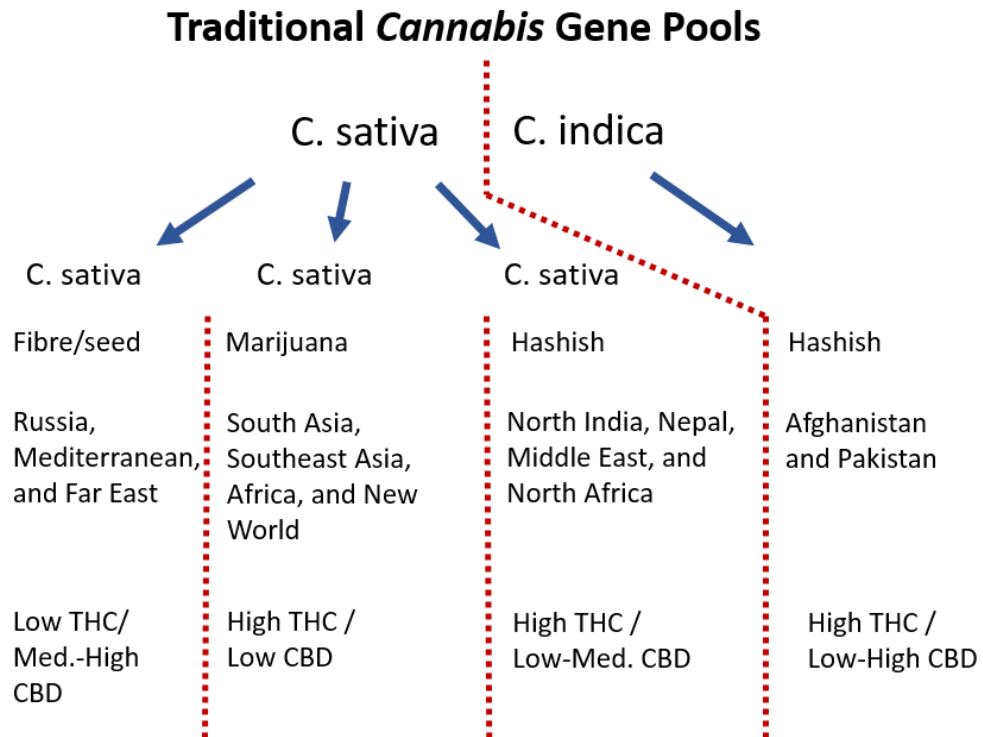


Figure 2-3 The four major cannabis gene pools. Adapted from (54)

### 2.1.2 Prevalence of cannabis use

The first archaeological evidence of cannabis use by humans was found in China roughly 4000 years B.C. (67). According to the 2016 World Drug Report by the United Nations Office on Drugs and Crime (UNODC), cannabis remains the most widely consumed drug worldwide by an estimated 183 million users (68). The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported in their latest publication on drugs trends that the prevalence of cannabis use is estimated to be five times that of other substances with cannabis being the most commonly used drug by approximately 84 million users. In Scotland, cannabis was reported by 20% of the 8,692 individuals providing information on recent 'illicit' drug use and seeking treatment (69).

### **2.1.3 Legal status of cannabis in the UK and KSA**

In the UK, four main statutes regulate the availability of drugs: the Medicines Act 1968, the Misuse of Drugs Act 1971, the Misuse of Drugs Regulations 2001, and the most recent Psychoactive Substances Act which came into force on 26 May 2016 as complementary to the 1971 act. The Misuse of Drugs Act 1971 is intended to prevent the non-medical use of certain drugs including cannabis (70), whereas the Misuse of Drugs Regulations 2001 intended to allow for the lawful possession and supply of controlled (illegal) drugs for legitimate purposes. Under the Misuse of Drugs Act 1971, illegal drugs are divided into Classes A, B and C, while in the Misuse of Drugs Regulations 2001, drugs are divided into five schedules. At the moment, cannabis is a class B, schedule 1 drug according to the UK legislations. It is illegal to possess, supply or produce this drug. Cannabis possession carries a maximum sentence of 5 years' imprisonment and a fine. Cannabis trafficking offences carry a maximum sentence of 14 years' imprisonment and a fine. It is noteworthy that based on the advice from the advisory council on the misuse of drugs, which was established to keep the drug situation under review, cannabis was downgraded from class B to class C in 2004 and was then upgraded back to class B in 2009 (70-72).

In the Kingdom of Saudi Arabia (KSA), narcotics control law differentiates between narcotics smugglers, dealers and users. For the first time offender, punishment is imprisonment, lashing or financial fine or all. For the repeat offenders, punishment is increased and the involved person may be sentenced to death. Narcotics users are jailed for two years and punished according to the judge's decision. If the offender is a foreigner, he is deported from the Kingdom. A narcotics user who enrolls in a treatment program is not questioned, but admitted into a specialized hospital (73).

#### **2.1.1 Modes of ingestion of cannabis**

Cannabis is available mainly in the form of herbal plant material or dried resin. According to the UNODC 2016 World Drug Report, Europe, North Africa and the Near and Middle East are the main markets for cannabis resin and is produced mainly in Morocco, Afghanistan and, to a lesser extent, Lebanon (68). In Saudi Arabia, official reports indicated that Yemen is an important source of cannabis

resin (74). Cannabis products have various street names including; hashish, bango, hemp, marijuana, pot, gandia, grass, chanvre and many more. The most potent form of cannabis is known as sinsemilla and is prepared from dried parts of mostly indoor-grown female plants. Typically, cannabis resin is smoked in combination with tobacco as joints. Smoking the joint involves burning the flowers and inhaling the active components of the plant that are released. Heat will lead to decarboxylation of the non-psychoactive precursor THCA-A to the primary active ingredient THC. Smoking is the dominant route of administration for illicit cannabis.

## 2.2 Cannabinoids pharmacology in humans

According to Dorland's Medical Dictionary, pharmacology can be defined as the science that deals with the origin, nature, chemistry, effects, and uses of drugs (1). The two main aspects of pharmacology are pharmacokinetics and pharmacodynamics. Pharmacokinetics describes what the body does to a drug following administration. Currently, it is defined as the study of the time course of drug absorption, distribution, metabolism, and excretion. Pharmacodynamics describes what a drug does to the body. It refers to the relationship between drug concentration at the site of action and the resulting effect, including the time course and intensity of therapeutic and adverse effects and involves receptor binding, post-receptor effects, and chemical interactions.

### 2.2.1 Pharmacokinetic of cannabinoids

The pharmacokinetic study of cannabinoids is known to be a challenging task due to several factors. Those contributing significantly to the problem are low analyte concentrations and rapid and extensive metabolism. Moreover, the physicochemical characteristics of cannabinoids restrain their separation from the biological matrices and from each other, and minimize drug recovery due to their adsorption into multiple body organs.

**Absorption** can occur through different sites in the body depending on the route of administration. Drugs that are inhaled or smoked, such as cannabis resin, are absorbed into the bloodstream through the lungs.  $\Delta^9$ -THC becomes detectable in plasma after just a few seconds following inhalation and reaches peak

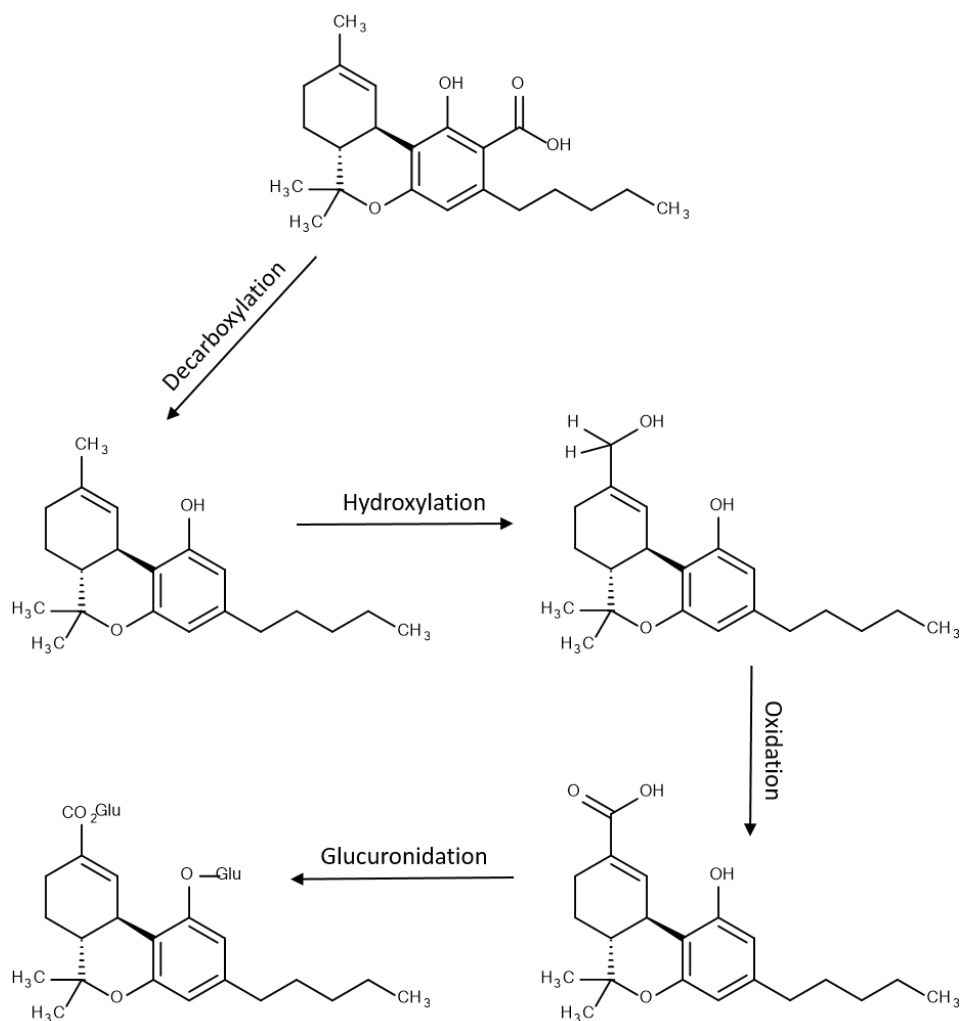
concentrations in 0.08 to 0.17 hour. After smoking, plasma concentrations for THC, 11-OH-THC and THC-COOH were found to peak in the range of 6-10 minutes, 9-23 minutes and 32-133 minutes, respectively (75,76). The maximal THC plasma concentration was found to be approximately 3-fold the maximal concentration of THC-COOH and 20-fold the maximal concentration of 11-OH-THC (76). However, after oral administration, the THC peak concentration in plasma is reached after 1 to 6 hours (77), which is a significant delay. The relatively long half-life is caused by the lipophilic nature of THC which enhances its distribution to adipose tissue and different body organs, then causes it to be slowly released back into the blood and metabolized (78,79). THC bioavailability was found to vary significantly according to inhalation behaviour during smoking and frequency of use. It was reported to range from 2-56% (80). Within 72 h after smoking, approximately 50% of the inhaled THC will be excreted as the metabolite, and the remaining 50% distributed throughout fatty tissue in the body.

Following absorption, several factors affect the degree to which drugs are **distributed** into body fat and tissues. These factors include the drug lipophilicity, protein binding, and the size of the drug. Higher volumes of distribution are reported for drugs which are more likely to be distributed widely into body fat or tissues. In blood, due to the low partition coefficient of the drug into red blood cells (RBCs), only 10% of  $\Delta^9$ -THC is distributed in the erythrocytes and about 90% is distributed into the plasma. More than 95% of THC in plasma is bound to plasma lipoproteins which complicates the THC initial disposition. The initial volume of distribution of THC was reported to be  $2.55 \pm 1.93$  L in drug-free users and  $6.38 \pm 4.1$  L in regular users, and 10 L/kg at steady-state (81).

Drug **metabolism** is the biochemical alteration of compounds through specialized enzymatic systems. The main purpose of metabolism is to prepare the drugs for excretion from the body, usually by making the drug more polar or converting the lipophilic compounds into more readily excreted hydrophilic products. Metabolism of some drugs will add some pharmacological activity by the formation of pharmacologically active metabolites or it may even be responsible for producing all the pharmacological activity following ingestion of a pro-drug. The liver is the primary organ responsible for metabolism but other sites including the kidneys are also involved. The  $\Delta^9$ -THC biotransformation takes place mainly in the liver and is

catalysed by enzymes of the cytochrome P450 (CYP) complex. In humans, more than 20 metabolites can be identified from  $\Delta^9$ -THC metabolism. In forensic toxicology,  $\Delta^9$ -THC metabolism leads to formation of two dominating metabolites of analytical interest. The first is 11-hydroxy- $\Delta^9$ -tetrahydrocannabinol (11-OH-THC), a product of microsomal hydroxylation of  $\Delta^9$ -THC. This metabolite is psychoactive and is further oxidized to 11-nor-9-carboxy- $\Delta^9$ -tetrahydrocannabinol (THC-COOH), the primary inactive metabolite (see Figure 2-4). It is noteworthy that there are several shortcuts used interchangeably throughout the literature to refer to this metabolite such as; 9-Carboxy-THC, THC-COOH, c-THC or THCA, however, THC-COOH only will be used in this work to refer to this metabolite.

**Elimination** is the process by which drugs are removed from the body. This occurs mainly by excretion through the kidneys, but some drugs may be eliminated in the bile, or via the lungs. The rate at which drugs are excreted from the kidneys will vary depending on the drug's properties, e.g. whether it is acidic (low pH) or basic (high pH), and on the pH of the urine. All aspects of the pharmacology of drugs should be taken into consideration when interpreting toxicological data (8). More than 65% of smoked THC is excreted in the faeces and about 20% in the urine (82,83). Usually THC-COOH is found in the urine as glucuronide conjugates while 11-OH-THC predominates in the faeces (84).



**Figure 2-4 The main metabolic route for  $\Delta^9$ -THC**

### 2.2.2 Cannabis mechanism of action

Due to the THC activity at many sites, scientists have encountered some difficulties in illustrating its mechanism of action. Over the years, two hypotheses were proposed for THC's mechanism of action. One hypothesis suggested that THC exerted its effects through non-specific interactions of the drug with cellular and organelle membranes (85,86). The other hypothesis suggested that THC interacted with specific cannabinoid receptors (87,88). However, our understanding of cannabinoid pharmacology has increased enormously over the last 25 years by several important discoveries. Central (CB1) (89,90) and peripheral (CB2) (90) cannabinoid receptors have been characterized, endogenous ligands (endocannabinoids); anandamide (91) and 2-arachidonyl glycerol (92), have been identified, and specific CB1 (93) and CB2 receptor antagonists (94) have been synthesized. The psychological and physiological effects of cannabis are

attributed, mainly, to the activation of these two main cannabinoid receptors by THC (95). CB1 receptors are found mainly on neurons in the brain, spinal cord and peripheral nervous system, whereas CB2 receptors occur principally in immune cells (95). The absence of cannabinoid receptors in the brain stem, which controls the body's vital functions may explain the low toxicity of cannabinoids. The activation of CB receptors on presynaptic cells by THC reduces the amount of neurotransmitter that gets released, which in turn affects how messages are sent, received, and processed by the cell.

### **2.2.3 Effects of cannabis**

Many factors contribute to the severity of cannabis use consequences. These factors include, but are not limited to, the dose and THC and CBD content in the plant, administration method, user's history, expectations and mood state (96). Effects of cannabis have been studied and reviewed extensively in the literature (96-100). Main cannabis effects include; euphoria, anxiety, altered time perception, loss of concentration, and panic attacks. The most commonly researched ones are feelings of well-being, euphoria, and relaxation. THC produces an increase in heart rate, blood pressure, and body temperature. It is also possible to experience dry mouth, increased hunger, and pain reduction. Very high doses of cannabis can cause anxiety, panic, or result in long-term impairment of memory and learning ability and psychotic episodes.

## **2.3 Analysis of cannabinoids in hair matrices**

The vast majority of studies have concentrated on identifying the most abundant cannabinoid analyte in hair, the more neutral and lipophilic parent compound, THC and its main metabolite THC-COOH, which is present in much lower concentrations in the hair. The main benefit of measuring THCCOOH in hair is that cannabis use can be more effectively documented than with detection of the parent THC which could be deposited in hair following environmental exposure to cannabis smoke. Analysis of cannabinoids in hair has challenged the sensitivity limits of immunoassay and confirmation assays; advanced instrumentations have been required in most cases to increase the confirmation rate of presumptive positive results.

In the latest version of guidelines of the Society of Hair Testing (SoHT) (26), the European Workplace Drug Testing Society (EWDTS) (101), the German Society of Toxicological and Forensic Chemistry (GTFCh) (102) and the Substance Abuse and Mental Health Services Administration (SAMHSA) (103), the following cut-off concentrations in Table 2-2 are recommended for detecting cannabis use in hair:

**Table 2-2 Recommended cut-offs for THC and THC-COOH in hair to identify use.**

Drug		Screening cut-off (ng/mg)	Confirmation cut-off (ng/mg)
SoHT & EWDTS	THC	$\leq 0.1$	0.05
	THC-COOH	-	0.0002
GTFCh	THC	$\leq 0.02$	-
	THC-COOH	-	-
SAMHSA	THC	-	-
	THC-COOH	$\leq 0.001$	0.00005

Despite the fact that a rapid, and simple gas chromatography mass spectrometry (GC/MS) method could be developed and employed to serve as a screening tool, the low cost of immunoassays makes it more preferable and widely utilized to screen for drugs of abuse to efficiently exclude negative samples and limit further confirmatory testing to presumptive positive samples.

### 2.3.1 Immunoassays

The term immunoassay is generally used to describe antibody-mediated analytical procedures. In these type of assays, the power of detection of antibody-antigen (Ab-Ag) reactions is exploited to develop assay technologies for detecting drug compounds and their metabolites in a variety of matrices. There are two main types of antibodies employed in immunoassays. The first type is polyclonal antibodies which are produced in-vivo inside different animals (such as sheep, goats) and contain a mixture of antibodies that have the capability to bind to different antigen binding sites (epitopes). The second type is monoclonal antibodies which are generated from a single cell line using hybridoma<sup>1</sup> technology

<sup>1</sup> **Hybridomas** are produced by injecting a specific antigen into a mouse, collecting an antibody-producing cell from the mouse's spleen, and fusing it with a tumor cell called a myeloma cell. The **hybridoma** cells multiply indefinitely in the laboratory and can be used to produce a specific antibody indefinitely



and mouse myeloma cell lines. These antibodies are known to be more specific for a single epitope on a multivalent antigen.

Immunoassays come in many different formats and variations, competitive and non-competitive, heterogeneous and homogeneous. In competitive formats, the analyte of interest (unlabeled antigen) in the test sample is measured by its ability to compete with the labeled antigen in the immunoassay. Immunoassays that require separation of bound and free labelled antigen are referred to as being heterogeneous immunoassays. Those that do not require separation are referred to as homogeneous immunoassays. Homogeneous methods have generally been applied to the measurement of small analytes such as drugs of abuse and therapeutic drugs. Examples of the most commonly reported immunoassays include radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), enzyme-multiplied immunoassay technique (EMIT), fluorescence polarization immunoassay (FPIA), cloned enzyme donor immunoassay (CEDIA) and kinetic interaction of microparticles in solution (KIMS). These tests have mainly been reported for screening drugs of abuse in urine samples and their principles usually implied in names.

#### **2.3.1.1 Immunoassays as a screening tool for hair matrices**

One of the concerns with regards to immunoassay screening for hair matrices is the incompatibility of immunoassay kits designed for biological fluids, such as urine, blood and oral fluid, with hair. This incompatibility arises either from sample pre-treatment variations or due to targeting different parent drugs or metabolites (104). The traditional method for extraction of incorporated drug from the hair matrix is overnight incubation at a certain temperature with organic solvent such as methanol (MeOH). This method has been proven to cause degradation in the performance of the immunoassay even with evaporation of methanol and reconstitution with buffer. Coulter *et al* have reported a successful method to overcome this by adaption of the sample pre-treatment step to make it more suitable for ELISA (105). In their work, an aqueous incubation, to extract drugs from the hair matrix, was used and the hair extract was directly placed into the ELISA microplate well (105). In addition to the improved sensitivity, less extraction time, elimination of evaporation and reconstitution steps were advantages from the new extraction protocol. Referring to this proposed

extraction method, Moore stated that ‘the method should expand the menu of immunoassay screening kits for drugs in hair matrices as the major obstacle no longer exists’ (106). In the same year, 2010, Comedical Laboratories marketed a (VMA-T) reagent purposely designed to treat hair samples for compatibility with immunological methods currently used in urinalysis. Its assays convert 6-monoacetylmorphine (6-MAM) and cocaine (which may be present in hair) to their metabolites which are detected in urine, specifically morphine and benzoylecgonine (107). Although cannabinoids immunological methods are not benefited directly from this reagent, a complementary reagent (M3), which has been more recently proposed from the same provider, was employed successfully for extraction of 11-nor-D9-tetrahydrocannabinol-9-carboxylic acid glucuronide (THC-COOH-glu) in hair for the first time by Pichini *et al* (108). Many laboratories are currently using ELISA tests, not specifically designed for hair testing but compatible with this matrix if the sample is prepared suitably (109-111). Alternatively, there are purposely designed immunoassays for hair testing analysis (112).

#### **2.3.1.2 Evaluation of immunoassay kits for the detection of cannabis using hair matrices**

Most commercially available cannabinoid immunoassay kits are calibrated with the major metabolite THC-COOH, as the presence of this metabolite is believed to prove consumption and excludes the possibility of external contamination which is always present when targeting only THC. The full validation of an immunoassay method is looked at as a very complex task. Many factors contribute to this complexity including; (1) nonlinearity of the calibration curves and the decisive consequences of that on the validity of the results at the cut-off value, and (2) the potential presence of unwanted cross-reactivities and unspecific binding to matrix components. The validation and cut-off value determination is generally carried out by the manufacturers. Therefore, the cut-off value decision is extremely important and has a direct impact on the detection time window and the positive rate. There are many methods reported in the literature for evaluating immunoassay performance. The most common one is achieved by measuring the following four parameters; true-positive (TP), true-negative (TN), false-positive (FP) and false-negative (FN) of the assay. Usually these can be obtained by comparing results of immunoassay screening and a confirmatory

technique such as GC/MS. Results are then used to calculate the specificity  $[(TN / (TN + FP))] \times 100\%$ , sensitivity  $[TP / (TP + FN)] \times 100\%$ , and efficiency  $[(TP + TN) / (TN + FP + TP + FN)] \times 100\%$ . Knowledge of the sensitivity and specificity of cannabinoid immunoassays for different cannabinoid analytes is essential for their proper use, since these assay characteristics differ and affect detection times. The performance characteristic of two immunoassay kits, one from NeoGen for THC-COOH and the other one obtained from Immunalysis for THC, were carried out. Both kits have shown good performance, however, the Immunalysis kit was found to be better for targeting THC while the NeoGen kit had the superiority with THC-COOH. A copy of two posters presenting the work and results are shown in Appendix I and Appendix II.

### 2.3.2 Confirmatory Testing

It is a common practice in forensic toxicology laboratories to carry out the confirmation technique by a chemical technique that is based on a different scientific principle from the chemical technique used in the initial test. Typically, chromatographic techniques coupled with mass spectrometry are used for confirmation due to their high specificity. Analysis of hair samples using these kind of techniques usually involve several steps including; extraction of drugs from inside the hair matrix, cleaning the hair extract, derivatisation (if required), and analysis using one of the following techniques; GC-MS, GC-MSMS, LC-MS, LC-MSMS. A review of sample preparation methods, instrumentations employed, commonly targeted analytes for cannabinoids and range of concentrations detected in hair is included in the following three sections.

#### 2.3.2.1 Sample preparation

The importance of sample preparation in analytical methods lies in its huge impact on sensitivity, through concentration of the target analyte and exclusion of unwanted interferences in the sample. In hair analysis, differentiation between external contamination and incorporated analyte in hair is another function of sample preparation, giving it an even more crucial role. Methods for the determination of cannabinoids in hair usually include the following basic steps: 1. washing hair with a solvent to remove any cannabinoids adsorbed to external surfaces of the hair; 2. digestion or dissolution of the keratinic matrix to extract

cannabinoids and metabolites from inside hair, this step is carried out usually using strong alkaline hydrolysis; 3. further cleaning or extraction of the digested hair; 4. derivatization of the extracted cannabinoids; and 5. analysis using gas or liquid chromatographic coupled with mass spectrometry.

So far, it is an acknowledged fact that no standardised washing procedures can effectively remove all external contaminants with no loss from the targeted analyte (113,114). Cannabinoids are present in cannabis smoke and passive exposure to this smoke can result in external adsorption of cannabinoids into the hair shaft. Therefore, determination of THC-COOH, the main metabolite for cannabis, in hair continues to be the main frequently used indicator to prove cannabis consumption.

Despite the fact that alkaline hydrolysis is the most favoured and dominated method for extraction of cannabinoids incorporated into hair, other methods have been reported in the literature, such as enzymatic hydrolysis (115,116), methanol (117,118), and acidic aqueous buffer (119), diatomaceous followed by pressurised liquid extraction (PLE) (120) and acetonitrile (121). Dissolving hair using alkaline hydrolysis involves using a strong base such as sodium hydroxide (NaOH), (122-127), or potassium hydroxide (KOH) (128,129). Alkaline hydrolysis is generally preferred over the other methods due to its efficiency, simplicity, and can be carried out very rapidly. However, the method is not suitable for all analyte and other methods must be used to extract these from hair. For instance, the ester-linked glucuronide conjugate (THC-COOH glucuronides) will be cleaved and converted back to THC-COOH when alkaline hydrolysis used. Also, heating is essential to accelerate the hydrolysis, and an analyte such as tetrahydrocannabinolic acid A (THCA-A) will decarboxylate to form THC when heated.

After extraction of analytes from within the hair, the resulting digest is not suitable for injection and needs further cleaning. One of the main challenges in cannabinoids analysis is the differences in chemical properties for the main cannabinoids THC, CBD and CBN and the main metabolite THC-COOH. This variation makes it difficult to, effectively and simultaneously, extract all compounds in one universal procedure. Therefore, it is important to adjust the pH of the hair digest prior to extraction of THC-COOH. Early methods for the

determination of cannabinoids in hair used liquid-liquid extraction (LLE) (115,130-133), solid-phase extraction (SPE) (114,120,134), solid-phase microextraction (SPME) (124,135,136), and solid-phase dynamic extraction (SPDE) (125). A two-step liquid/liquid extraction procedure seems to be the most used and reported cleaning method. An extraction solvent is added to the hair digest without prior manipulation to extract the main cannabinoids (THC, CBD, CBN) and the metabolite 11-OH-THC, the aqueous layer is then separated, acidified or neutralised, and re-extracted with the same solvent or different extraction method, such as SPE. Varying volumes of acetic acid are most often reported for this purpose (137-139), other acids have been reported as well, such as acetate buffer with acetic acid (140,141), hydrochloric acid (129), formic acid (142) and maleic acid (138). The two organic extracts are then either combined or prepared separately for derivatisation or straight analysis. Different extraction solvents were reported for extraction of cannabinoids using LLE; n-hexane-Ethyl acetate (EtOAc) (90:10, v/v) (130,131,138,140,143), hexane-EtOAc (3:1, v/v) (133), Ethyl Acetate (EtOAc) (142), pentane (116), chloroform / isopropyl alcohol (97:3, v/v) (115).

A common method to analyse cannabinoids involves gas chromatography-mass spectrometry (GC-MS). In most cases, derivatisation is often utilised to make the analytes volatile and improve chromatographic and mass spectrometric (MS) identification. Following review of the literature for cannabinoids analysis in hair, it was found that the developed analytical methods either do not include a derivatisation step (116,124,132,136,144), or use a silylation derivatisation agent (such as BSTFA or MSTFA) (65,114,125,130,133-135,145), or acylation reagents (usually a mixture of a perfluorinated anhydride (TFAA, PFPA, or HFBA) and a perfluorinated alkyl alcohol (HFIP or PFPOH) (114,115,134,137,138,140,146,147).

#### 2.3.2.2 Analysis

Cannabinoids are not incorporated into hair as efficiently as most other drugs. As a result, concentrations of cannabinoids in hair after smoking or ingestion of cannabis are very low and can only be detected with extremely sensitive analytical methods. Several methods for hair analysis of cannabinoids have been developed using gas chromatography mass spectrometry operating in electron impact mode

(GC-EI-MS) (65,115,116,125,132,133,135,145,148,149), gas chromatography mass spectrometry in negative chemical ionization mode (GC-NCI-MS) (123,138,150-152), gas chromatography tandem mass spectrometry in negative chemical ionization mode (GC-NCI-MSMS) (134,139,140,143,147,149,153-156), gas chromatography tandem mass spectrometry in positive chemical ionisation mode (GC-PCI-MSMS) (134), two-dimensional gas chromatography time-of-flight mass spectrometry in electron impact mode (157), two-dimensional gas chromatography mass spectrometry in negative chemical ionization mode (2D GC-NCI-MS) (141), or two-dimensional gas chromatography tandem mass spectrometry in EI mode (2D GC-EI-MS) (158). The analytical procedures described in the literature for the analysis of cannabinoids in human hair are mainly targeted for the simultaneous detection of parent cannabinoids THC, CBD and CBN (107,119,124-127,132,133,135,136,144,145,148,159), the main metabolite THC-COOH only (123,139,141,143,151,152,154,158,160-163) or, both, one or more of parent cannabinoids (THC, CBD and CBN) and the main metabolite (THC-COOH) (115,120,130,131,164). Simultaneous detection of both parent cannabinoids and the main metabolite from hair is a very challenging task. The differences in chemical properties of the metabolites make it difficult to use one universal extraction procedure to extract all cannabinoids efficiently in one single elution. Usually, the pH of the hair digest with sodium hydroxide needs to be adjusted after extraction of the parent cannabinoids (THC, CBD and CBN) and prior to the extraction of THC-COOH. This process ends up in two fractions. Combining the two fractions makes derivatisation more complicated. Due to the extremely low concentrations of THC-COOH in hair, perfluorinated agents are often preferred due to the superior sensitivity that can be achieved when this derivative is analysed using MS operated in NCI mode. However, derivatisation of THC and CBD with perfluorinated agents will produce identical retention times and identical mass spectra, and hence will result in inaccurate quantitation results. This problem was highlighted previously in the literature for PFPOH-PFPA (115) and HFIP-TFAA (165). Derivatisation of all cannabinoids with silylation agents such as BSTFA or MSTFA will solve this problem and produce derivatives that can be separated when analysed on standard GC-MS instrumentation in EI mode. However, the problem exists that although the concentrations of THC, CBD and CBN are present in hair in the nanogram range and GC-EI-MS may provide sufficient sensitivity to detect them, the concentrations of THC-COOH are present in hair in

the picogram range which cannot be detected on standard GC-EI-MS. It would appear that there is no GC-based method in the literature allowing the simultaneous determination of THC, CBD, CBN and THC-COOH in one sample preparation and injection. Recently, Dulaurent *et al* reported a liquid chromatography tandem mass spectrometry (LC-MSMS) method for the determination of the four compounds in one sample preparation and injection for the first time(131). The other methods describing the determination of the 4 compounds used 2 sample preparations and 2 injections (114,115) or 1 sample preparation and 2 injections (134). Tandem MS operated in NCI is often employed to target THC-COOH in hair and is known to provide good sensitivity. However, due to the high cost of such instrumentation, 2D GC-NCI-MS is proposed as a cheaper alternative.

#### **2.3.2.3 Concentrations of cannabinoids in hair**

In general, there is an accepted consensus that concentrations of THC, CBD and CBN are in the high picogram to low nanogram per milligram of hair range, while concentrations of THC-COOH are in the high femtogram to low picogram per milligram of hair range. There have been several studies investigating the concentrations of different cannabinoids in hair. The range of concentrations detected in these studies is compared against the concentrations obtained in this thesis and shown in detail in section 5.8.3.

## Chapter 3 Hair sample preparation and extraction methods for THC, CBD, CBN, 11-OH-THC, and THC-COOH

### 3.1 Introduction

In this project, only the main cannabinoids (THC, CBD, CBN) and phase-one main metabolites (11-OH-THC and THC-COOH) were targeted, therefore, NaOH was employed to extract analytes of interest from the hair. Despite the fact that the n-hexane/ethyl acetate mixture is the most frequently reported extraction solvent in the literature, for health and safety reasons, n-hexane use is restricted for routine analysis in the Forensic Medicine and Science laboratory (FMS), University of Glasgow due to its potential carcinogenicity and chronic toxicity effect. As an alternative to n-hexane and aiming at optimisation of the extraction method, the extraction power of five solvents for cannabinoids from hair; cyclohexane, cyclohexane-EtOAc (90:10, v/v), methyl tert-butyl ether (MTBE), ethyl acetate (EtOAc), and dichloromethane (DCM) was investigated. Acidification of the hair digest is another area that was subject to further investigation. With exception of maleic acid, which was not readily available, a series of experiments were carried out to evaluate the effect of all the acids reported in the literature for pH adjustment on the recovery of THC-COOH. In addition to LLE of hair for THC-COOH, two different extraction methods were evaluated; supported-assisted liquid-liquid extraction (SALL) and solid-phase extraction (SPE). SALL evaluation was carried out following a protocol proposed by the manufacturer. The initial method for SPE was based on previously published work by Moore *et al* (141). Due to an issue during extraction, the SPE method was subject to further optimisation. The extraction comparison will be discussed further in sections 3.5 and 3.6. Traditionally, the most common derivatising reagents used for analysis of THC-COOH on GC-MS with negative chemical ionisation (NCI) are the fluorinated anhydrides. The need for derivatisation becomes important especially when targeting the main metabolite (THC-COOH), which is more polar and has a high boiling point and will degrade at high temperatures. The chemical structure of THC-COOH provides two possible sites for derivatisation; carboxyl and hydroxyl groups. With the aim of optimising the detection sensitivity, both groups were subject to different combinations of derivatisation.



## 3.2 Aims

The purpose of the work presented in this chapter was to optimise extraction methods for the three parent cannabinoids (THC, CBD, and CBN) and the two metabolites (11-OH-THC and THC-COOH) in hair samples. In addition, the outcome of different derivatisation reagent combination for THC-COOH was investigated. Five extraction solvents including; EtOAc, cyclohexane, methyl tert-butyl ether and dichloromethane, were tested as alternative extracting solvents to n-hexane for liquid-liquid extraction (LLE). The optimised method giving the highest recoveries would then be selected to analyse these drugs in hair. Different methods for the purpose of acidifying the alkaline hair digest sample were compared for the extraction of THC-COOH using LLE. Moreover, SALL and SPE extraction methods were compared for the extraction of THC-COOH.

## 3.3 Materials and methods

### 3.3.1 Materials

$\Delta^9$ -Tetrahydrocannabinol (THC) at 100  $\mu\text{g/mL}$ , Cannabidiol (CBD) at 1  $\text{mg/mL}$ , Cannabinol (CBN) at 1  $\text{mg/mL}$ , 11-Hydroxy- $\Delta^9$ -tetrahydrocannabinol (11-OH-THC) at 100  $\mu\text{g/mL}$ , and 11-nor-9-carboxy- $\Delta^9$ -tetrahydrocannabinol (THC-COOH) at 100  $\mu\text{g/mL}$  were purchased from Sigma Aldrich (Basingstoke, UK). All of these drugs were purchased dissolved in 1 mL methanol. Deuterated internal standards (ISTD);  $\Delta^9$ -Tetrahydrocannabinol- $d_3$  (THC- $d_3$ ), 11-Hydroxy- $\Delta^9$ -tetrahydrocannabinol- $d_3$  (11-OH-THC- $d_3$ ) and 11-nor-9-carboxy- $\Delta^9$ -tetrahydrocannabinol- $d_3$  (THC-COOH- $d_3$ ) (100  $\mu\text{g/mL}$ ), each at 100  $\mu\text{g/mL}$ , were obtained from Sigma Aldrich (Basingstoke, UK). The following derivatisation reagents; N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), trifluoroacetic anhydride (TFAA), pentafluoropropionic anhydride (PFPA), 2,2,3,3,3-Pentafluoro-1-propanol (PFPOH), Heptafluorobutyric anhydride (HFBA), and Methyl iodide were purchased from Sigma Aldrich (Basingstoke, UK). The following chemicals; acetonitrile (ACN), glacial acetic acid, methanol (MeOH), ethyl acetate (EtOAc), dichloromethane (DCM), hexane, cyclohexane, methyl-t-butyl ether (MTBE), and hydrochloric acid 37% (HCl) were supplied by VWR International Ltd, (Lutterworth, UK). Deionised water was obtained from the in-house Millipore® system. ChemElut-1mL

unbuffered supported-assisted liquid-liquid extraction (SALL) extraction cartridges was obtained from Agilent, UK

### 3.3.2 Preparation of chemical solutions

#### 3.3.2.1 0.1M sodium acetate buffer

2.93g of sodium acetate trihydrate was weighed in a weighing boat, transferred into a beaker and then dissolved in 400 mL deionised water. 1.62 mL glacial acetic acid was then added. The buffer was pH adjusted to pH 4, 5 or 6 with 0.1M acetic acid (to lower pH) or 0.1M sodium acetate (to raise pH). After adjusting the pH, the solution was then transferred into a 500 mL volumetric flask, and made up to the mark with deionised water. This was mixed well and stored at approx. 5°C and discarded after 30 days.

#### 3.3.2.2 0.1 M hydrochloric acid

4.2 mL of concentrated hydrochloric acid (37.5% concentrated HCl) was added slowly to a 500 mL volumetric that was already filled with about 400 mL of deionised water (DI H<sub>2</sub>O). The volume was then made up to the mark with deionised water and stored at room temperature.

#### 3.3.2.3 1 M Acetic Acid

28.6 mL of glacial acetic acid was added into a 500 mL volumetric flask that was approximately half filled with DI H<sub>2</sub>O and mixed. The total volume was then brought up to 500 mL with DI H<sub>2</sub>O. The solution was stored at room temperature and discard after two months.

#### 3.3.2.4 1 M Sodium hydroxide

One mole of NaOH (40 g) was added slowly, with constant stirring and monitoring the temperature of the solution, to about 750 ml of deionized water in a 1 litre beaker. After all the NaOH has been added, the solution was transferred to a 1 L volumetric flask and made up to the mark with deionized water. The solution was then stored in a plastic container with plastic stopper at room temperature and discarded after two months.

#### **3.3.2.5 Cyclohexane (or n-hexane) / EtOAc (9:1, v/v)**

A ratio of 9:1 (cyclohexane (or n-hexane): ethyl acetate) was used. The volume of extracting solvent was prepared dependent on the number of samples to be extracted at the time of analysis.

#### **3.3.2.6 Cyclohexane / EtOAc (3:1, v/v)**

A ratio of 3:1 (cyclohexane:ethyl acetate) was used. The volume of extracting solvent was prepared dependent on the number of samples to be extracted at the time of analysis.

#### **3.3.2.7 0.1M HCl / ACN (70:30, v/v)**

A ratio of 7:3 (0.1M HCl:ACN) was used. The volume of SPE washing solution was prepared dependent on the number of samples to be extracted at the time of analysis.

### **3.3.3 Preparation of cannabinoid working solution mix**

Individual stock solutions of THC, 11-OH-THC and THC-COOH were prepared at a concentration of 10 µg/mL from purchased reference materials at 100 µg/mL by 1:10 dilution with methanol. Stock solutions of CBD and CBN were prepared at a concentration of 10 µg/mL by 1:100 dilutions with methanol from 1 mg/mL reference standards. A working solution mixture at 1 µg/mL for analytes including (THC, CBD, CBN, 11-OH, and THC-COOH) was prepared by 1:10 dilution of stock solutions at 10 µg/mL.

### **3.3.4 Preparation of cannabinoid internal standard mix**

Individual stock solutions of internal standards THC-d3, 11-OH-THC-d3 and THC-COOH-d3 were prepared at a concentration of 10 µg/mL from purchased reference materials at 100 µg/mL by 1:10 dilution with methanol. One Internal standards mixture at 1 µg/mL containing THC-d3, 11-OH-THC-d3, and THC-COOH-d3 was then prepared by 1:10 dilution of stock solutions at 10 µg/mL.

### 3.3.5 Instrumentation

The instrument parameters employed to carry out extraction recovery studies and derivatisation are briefly described in this chapter. The method development process, initial methods testing and the analytical principles will be covered in extensively in the following chapter. The two analytical systems used are gas chromatography mass spectrometry (GC-MS) (system1) and two-dimensional gas chromatography mass spectrometry (2D GC-MS) (system2). GC-MS (system1) was used for analysis of TMS derivatives of THC, CBD, CBN, 11-OH-THC and THC-COOH. Analysis was carried out using an Agilent gas chromatograph (6890) equipped with single quadrupole mass spectrometry (5795). Chromatographic separation was performed using a DB-5MS DG (30 m x 0.25 mm ID, 0.25 µm film thickness) column (Agilent J&W). The mass spectrometer was operated in the selected ion monitoring (SIM) mode with electron impact ionisation at an electron energy of 70 eV. This instrument was employed to carry out most of the recovery studies. 2D GC-MS (system2) was employed to analyse the fluorinated derivative of THC-COOH. Analysis was carried out using an Agilent gas chromatograph (6890) equipped with single quadrupole mass spectrometry (5795). Chromatographic separation was achieved using two capillary columns: a DB-5MS ultra inset (30 m x 0.25 mm ID, 0.25 µm film thickness) column as primary column and a HP-17MS (15 m x 0.320 mm ID, 0.25 µm film thickness). The mass spectrometer was operated in the negative ion chemical ionization mode using high purity ammonia as the reagent gas. MS identification was carried out using SIM mode. This instrument was employed to carry out the recovery studies from Supported-Assisted liquid-liquid extraction (SALL), monitor drug loss experiment, and to test different derivatisation reagent combination for the THC-COOH.

### 3.3.6 Blank hair

Blank hair was obtained from two sources known to be drug free; (1) volunteers within Forensic Medicine and Science (FMS), University of Glasgow, (2) friends and family members. Hair was first washed according to the wash protocol of FMS. Hair was then screened separately for the presence of interferences by the GC-MS method. No signals were observed at the retention times of targeted ions. Hair that screened negative and had no interferences was pooled together and mixed before weighing out 40-50 mg aliquots for extraction recovery studies.

### 3.3.7 Digestion (Alkaline hydrolysis)

40-50 mg of blank hair was spiked with different volumes of analytes depending on the purpose of the experiment. The exact volumes are detailed in the experimental sections. 1N NaOH sodium hydroxide (1 mL) was added, and the hair was heated at 70°C for 30-60 min. The sample was allowed to cool down to room temperature before carrying out further clean-up steps.

### 3.3.8 Extraction recovery calculations

Recovery is defined as ‘the extraction efficiency of an analytical process, reported as percentages of the known amount of analytes of interest which are extracted and analysed by an optimised method’ (166) . The loss of analyte during extraction should be investigated with at least three replicates at two quality control (QC) levels. Internal standard is added after extraction to allow direct comparison with neat un-extracted standards. In order to calculate the recovery percentage, a non-extracted standard at the same concentration was also prepared at the same time in triplicate. Internal standard solution was added after the extraction to all extracted and non-extracted standards. The peak area ratios of the analyte to its respective deuterated ISTD were calculated. The absolute recovery was determined for each analyte by dividing the extracted STD/ISTD ratio by that of the non-extracted STD/ISTD ratio at the same concentration and multiplying by 100. This method was applied to all experiments. Ions that were used to calculate extraction recoveries are shown in tables Table 3-1 and Table 3-2.

**Table 3-1 Ions acquired in the Selected Ion Monitoring (SIM) mode using system1**

SIM group	Analyte	m/z	Internal standard	m/z
1	CBD -2-TMS	390	THC- <i>d</i> 3-TMS	374
2	THC-TMS	371		
3	CBN-TMS	367		
4	11-OH-THC-2-TMS	371	11-OH-THC- <i>d</i> 3-2-TMS	374
5	THC-COOH-2-TMS	371	THC-COOH- <i>d</i> 3-2-TMS	374

**Table 3-2 Ions acquired in the Selected Ion Monitoring (SIM) mode using system2**

SIM group	Analyte	m/z	Internal standard	m/z
1	THC-COOH-TFAA-HFIP	422	THC-COOH- <i>d</i> 3-TFAA-HFIP	425

### 3.4 Liquid-Liquid extraction

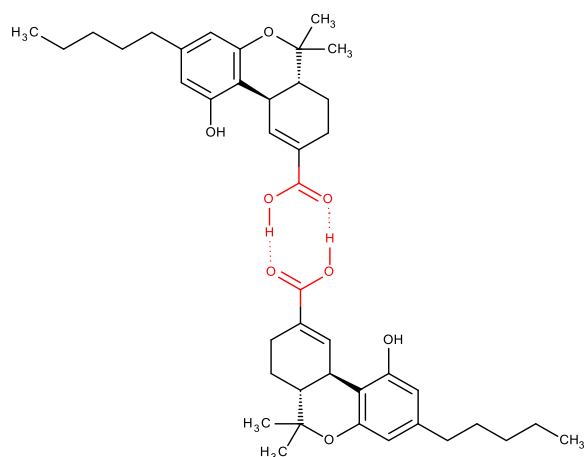
#### 3.4.1 Rationale for LLE

Liquid-liquid extraction (LLE) is an extraction of a substance from one liquid into another liquid phase. It consists in transferring one (or more) solute(s) contained in an aqueous solution to another immiscible organic liquid (solvent). It is also known as solvent extraction and partitioning, as the method to separate compounds is based on their relative solubility in the two immiscible liquids. One of the factors that determine drugs solubility is their ionisation status. As a rule of thumb, drugs are more hydrophilic when present in its ionised forms than the unionized forms because of the hydration of the ions, therefore the ionized drugs are difficult to extract into organic solvents whereas the unionized forms will dissolve in the organic solvents which can be extracted into organic solvents. For hair analysis, all cannabinoids are extracted from the hair into alkaline solution after addition of 1M sodium hydroxide (NaOH) during sample preparation prior to LLE. The pH of hair alkaline solution is about 13-14.

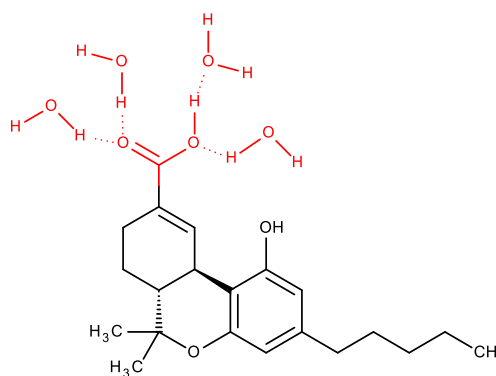
According to Henderson-Hasselbach Equation, any drug will be either in its 100% ionised or 100% unionized state when two pH units away from its pKa, higher pH than pKa promotes ionisation. The pH of aqueous solutions in which a drug is dissolved determines the form in which the drug is present. For example, THC-COOH has a pKa value of 4.2. When present in a hair digest solution with pH 14, it will be 100% ionized. All five cannabinoids, which are targeted for isolation by

LLE, are believed to be in their ionised forms under these very basic conditions. However, it has been reported frequently in the literature that direct extraction of the hair digest solution into a n-hexane:ethyl acetate mixture results in moving THC, CBD, CBN and 11-OH-THC from the digested hair solution into the organic solvent layer 'Fraction (A)'. The carboxylated metabolite THC-COOH is generally extracted separately after acidifying the hair digest solution 'Fraction (B)'. Based on the fact that the ionised status of THC, CBD, CBN and 11-OH-THC did not prevent them from being more soluble in the organic solvent, there must be another reason that THC-COOH is retained in the aqueous layer.

The carboxylic acid group present on the THC-COOH structure is considered to be a highly polar organic functional group. This polarity results from the presence of a strongly polarized carbonyl (C=O) group and hydroxyl (O-H) group. The O-H group here is even more strongly polarized than the O-H group of alcohols due to the presence of the adjacent carbonyl moiety. These structural features not only enhance dipole strength, but also are responsible for the acidity of THC-COOH. Also, as a result of the ability to form "solubilizing" H-bonding interactions with like molecules and water as shown in Figure 3-1 and Figure 3-2, THC-COOH displays relatively high water solubility compared to the other cannabinoids.



**Figure 3-1 Intermolecular H-Bonding**



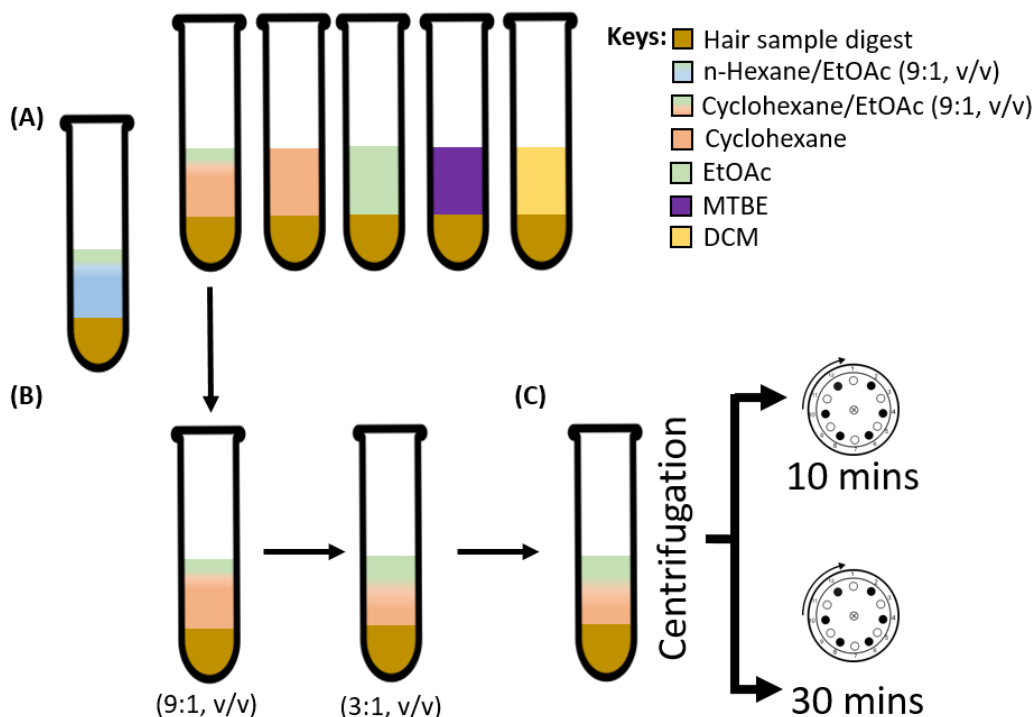
**Figure 3-2 H-Bonding with Water**

For LLE 'Fraction (A)', three main areas, as shown in Figure 3-3, were subject to investigation. Firstly, selection of the extraction solvent. Properties of proposed alternative solvents are shown in Table 3-3. In addition, DCM and a mixture of cyclohexane/ethylacetate (9:1, v/v) was prepared and included in the comparison. The choice of dissolving agent may become an issue in the process of refining a method. A range of solvents were assessed in order to determine which one extracted the most cannabinoids. Several factors must be considered while choosing a solvent to extract a drug from the matrix in addition to its powder to dissolve the required compounds which include selectivity, density, toxicity, volatility, reactivity, physical hazards and miscibility with aqueous media. Five solvents were examined as potential alternatives to n-hexane due to health and safety concerns related to it. Percentage of recovery and cleanness of extract were the main criteria of selection. Secondly, optimisation of selected solvent composition. As the selected solvent was a mixture of two solvents, the selected solvent composition was subject to further optimisation. Thirdly, the effect of centrifugation duration on extract cleanness.

**Table 3-3 Solvents properties**

Solvent	Formula	Density (g/mL)	Sol. in H <sub>2</sub> O (% at 20 °C)	Relative polarity
cyclohexane	C <sub>6</sub> H <sub>12</sub>	0.779	0.005	0.006
ethyl acetate	C <sub>4</sub> H <sub>8</sub> O <sub>2</sub>	0.894	8.7	0.228
methyl t-butyl ether (MTBE)	C <sub>5</sub> H <sub>12</sub> O	0.741	4.8	0.124





**Figure 3-3** A diagram showing areas of optimisation for LLE 'Fraction (A). (A) selection of extraction solvent, (B) optimisation of selected solvent composition, and (C) optimisation of sample centrifugation duration after LLE.

For LLE 'Fraction 'B', the THC-COOH is isolated into organic solvent by converting it from its water soluble form to the insoluble non-ionic acid form by acidification. As different acidification methods were proposed in the literature, the most frequently reported methods were used and compared in this study.

### 3.4.2 THC, CBN, CBD, 11-OH-THC (Fraction 'A')

#### 3.4.2.1 Experimental - Selection of extraction solvent

Selection of extraction solvent was carried out in two stages. Firstly, extraction recoveries of drugs of interest from the spiked aqueous digestion solution (1mL 1M NaOH) without involving hair matrices were calculated. Later, 40-50 mg of hair was weighed out and spiked with the same concentrations of analytes. For both recovery experiments, four sets of the following two levels were prepared; 200 and 400 ng of THC, CBD, CBN and 11-OH-THC. Alkaline hydrolysis was carried out according to the conditions described in section 3.3.7. Hair digest solution was first left to cool at room temperature and then centrifuged for 10 min at 2500 rpm and

supernatant was then transferred to clean glass vial. 1.5 mL of the extracting organic solvent, methyl-t-butyl ether, ethyl acetate, cyclohexane, cyclohexane:ethylacetate (9/1, v/v), DCM were added. To standardise the process, the sample was then placed on a rotator for 5 min at speed 25 rpm instead of manual shaking (see Figure 3-4). The mixture was then centrifuged for 10 min at 2500 rpm to allow the two layers to separate. The organic phase was removed using a pasteur pipette to a clean 7 mL vial. Prior to evaporation, 200 ng of internal standards, THC-*d*3 and 11-OH-THC-*d*3, were added and evaporated to dryness under a gentle stream of nitrogen at room temperature. Derivatisation was achieved with 30  $\mu$ L of BSTFA + 1%TMCS at 80 °C for 20 mins. Vials were then left to cool down at RT for 5 minutes and transferred to GC vial for injection.

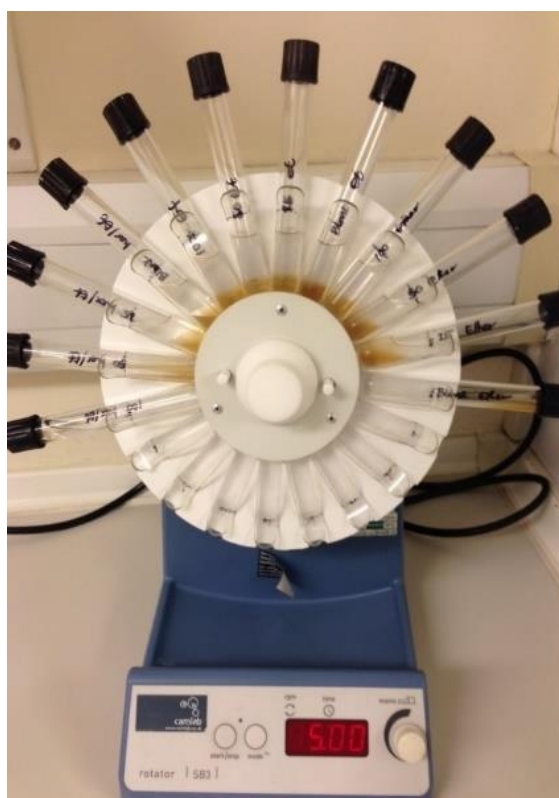


Figure 3-4 Rotator

#### 3.4.2.2 Experimental - Optimisation of solvent composition

The solvent composition is important to simultaneously extract THC, CBD, CBN and 11-OH-THC from hair. For selection of the optimal solvent composition, extraction recoveries of cyclohexane: EtOAc at two compositions (9:1, v/v) and (3:1, v/v) were calculated in triplicate at two different levels; 200 and 400 ng. LLE was carried out according to the procedure described in section 3.4.2.1

### 3.4.2.3 Experimental - Effect of centrifuge duration

The centrifuge is used to separate components of a mixture on the basis of particle size or density. In LLE, centrifugation is employed to separate the two immiscible layers and precipitate any unwanted particles suspended in the organic layer. Since the organic layer will contain interferences, increasing the centrifugation time to obtain cleaner extracts, was proposed. Two different centrifugation durations, 10 and 30 minutes, were assessed. Visual observation of the extract, chromatographic baseline and noise, and extraction recoveries were the assessment criteria. Analyses were carried out in triplicate at two levels, 200 and 400 ng, and the average calculated. LLE was carried out according to the procedure described in Section 3.4.2.1 using the optimal solvent composition (cyclohexane: EtOAc (3:1, v/v)).

### 3.4.3 THC-COOH (Fraction 'B')

#### 3.4.3.1 Experimental - Selection of sample acidification methods

After optimisation of the LLE procedure for analytes extracted in fraction 'A', the remaining cannabinoid THC-COOH had to be extracted from fraction 'B'. As it was necessary to adjust the pH prior to carrying out the second cycle of LLE, the literature was reviewed for the reported acidification methods. Different volumes of the following four acids were the most frequently reported:

- A. Acetic acid (114,115,131,137,138)
- B. Acetate buffer with Acetic acid (140,141,147)
- C. Formic acid (142,164)
- D. Hydrochloric acid (HCl) (150,167)

Following a review of the literature, it was found that the influence of acidification of the basic hair digest on the THC-COOH extraction recovery has never been reported. 120 µl acetic acid, 500 µl acetic acid, 1000 µl acetic acid, 1mL of 0.1M sodium acetate buffer (pH 4.5) and 200 µl acetic acid, 50 µl formic acid, and 100 µl HCl were used for acidification and compared against each other. Three sets of 40-50 mg fortified hair matrix with 200 and 400 ng of THC-COOH were prepared for each acidification method. Firstly, the prepared samples were subject to LLE using cyclohexane:EtOAc (3/1, v/v) as described in section 3.4.2.1.

The, remaining aqueous layer was acidified using the acids described above. A second cycle of LLE using 1.5 mL of cyclohexane:EtOAc (3/1, v/v) was carried out. The sample was then placed on a rotator for 5 min at speed 25 rpm and centrifuged for 10 min at 2500 rpm. The organic layer was transferred to a clean 7 mL vial. Prior to evaporation, 200 ng (200 µl of 1 µg/mL) of internal standard, THC-COOH-*d*<sub>3</sub>, was added and evaporated to dryness under gentle stream of nitrogen. Derivatisation was achieved with 30 µl of BSTFA with 1%TMCS at 80 °C for 20 mins. The vial was cooled down to RT for 5 minutes and transferred to GC vial for injection.

### 3.4.4 Simultaneous extraction of all cannabinoids using LLE

#### 3.4.4.1 Rationale

The extraction method that will, simultaneously, extract all cannabinoids is preferable when an advanced analytical instrument has the capabilities to achieve the required sensitivity for all analytes. The ionisation status of THC-COOH is important for analyte solubility, therefore, it was investigated whether an early acidification of the basic hair digest solution would be a suitable method for simultaneous extraction of all cannabinoids in a single fraction. Mercolini *et al* and Shah *et al* reported an early acidification of the basic hair digest solution, with formic acid and HCl, respectively, to extract THC and THC-COOH simultaneously in one fraction using LLE (142,167).

#### 3.4.4.2 Experimental

The basic hair digest solution was acidified using 100 µl HCl in three sets of hair samples spiked with 200 and 400 ng. The mixture was then extracted using cyclohexane: ethyl acetate (3/1, v/v) and derivatised according to the procedure described in the section 3.4.2.1.

### 3.5 LLE versus SALL for THC-COOH extraction

#### 3.5.1 Rationale for SALL

Due to the low concentrations targeted for THC-COOH in hair, an extraction method with an extremely clean output and high recovery is required. The conventional LLE does not provide the desired extract cleanliness. A SALL method was proposed as an efficient alternative to traditional LLE for extraction of THC-COOH in fraction (B). This method is claimed to provide high analyte recoveries, cleaner extracts, no emulsion formation, and significant reduction in sample preparation time. SALL utilizes the same water immiscible solvent systems for analyte extraction. In SALL, the aqueous sample is immobilised on an inert support, and the organic phase flows through the support instead of shaking the two immiscible phases together. A general SALL protocol is shown in Figure 3-5. Unlike SPE, the entire loaded sample is absorbed onto the extraction bed and does not go to waste. Therefore, the capacity of the SALL is critical and the sample volume to be loaded should not exceed the recommended limit to be able to absorb the whole sample.

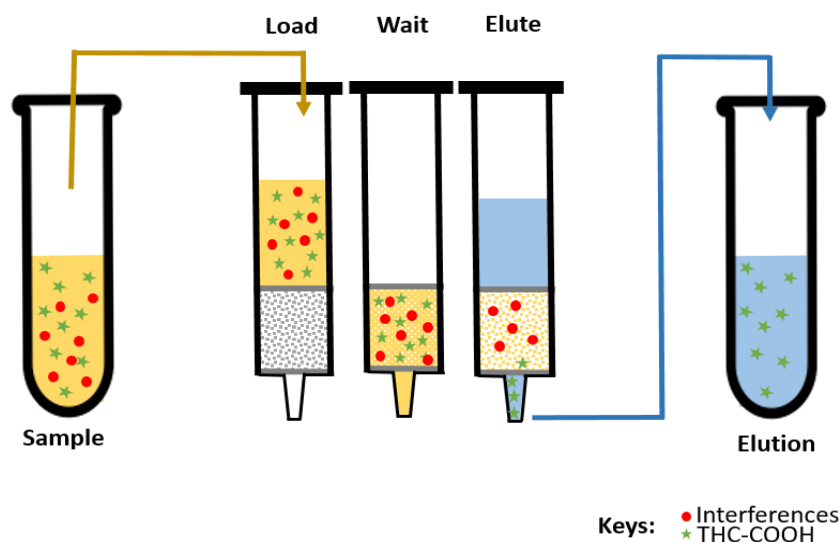


Figure 3-5 Extraction procedure for THC-COOH using SALL cartridges.

#### 3.5.2 Experimental

Two sets of 40-50 mg hair were prepared to compare a traditional LLE method against SALL. Each set was composed of triplicate samples for three levels at 1,

10 and 100 ng of THC-COOH. The hair was then digested with 800 µl of 1M NaOH. As the SALL sorbent was unbuffered, the pH of pre-treated hair digest was adjusted by adding 200 µl of acetic acid. Sample was loaded onto the column (and a pulse of vacuum was applied for 3-5 seconds to initiate the flow. The sample was then allowed to completely absorb for 5 minutes. Cyclohexane/EtOAc (3:1 v/v, 2x0.75mL) was then added and allowed to flow under gravity for 5 minutes. Organic solvent extract was collected and 10 ng of internal standard THC-COOH-*d*<sub>3</sub> was added prior to allowing it to dry under gentle stream of nitrogen. Traditional LLE extraction was carried out according to the procedure described in section 3.4.2.1 using 1.5 mL of cyclohexane:EtOAc (3:1, v/v). Extract was then derivatised by adding 30 µl TFAA-HFIP and incubating in heating block at 80 °C for 30 minutes.

## **3.6 Solid-Phase extraction for THC-COOH**

### **3.6.1 Rationale for SPE**

Solid-phase extraction uses siliceous or other materials with specific particle size (usually from 15 to 100 µm) in disposable plastic syringe barrels permitting sequential extraction, clean-up, and finally reproducible elution of THC-COOH at relatively low pressures. The employed SPE cartridge (CleanScreen® ZSTHC020) is copolymerized on a rigid, purified silica gel support. The two functional groups include a reverse phase, and an ion exchanger, primary amine. In anion-exchange SPE, the retention mechanism is the interaction of charged, anionic groups on the THC-COOH and charged, cationic functional groups on the sorbent, via ionic interactions as illustrated in Figure 3-6. SPE was proposed as an alternative extraction method to LLE and SALL for the remaining aqueous part (fraction 'B') of sample (A) or sample (B). Initially, the SPE method was investigated using a protocol based on previously published work for THC-COOH extraction from hair by Moore *et al* (141).

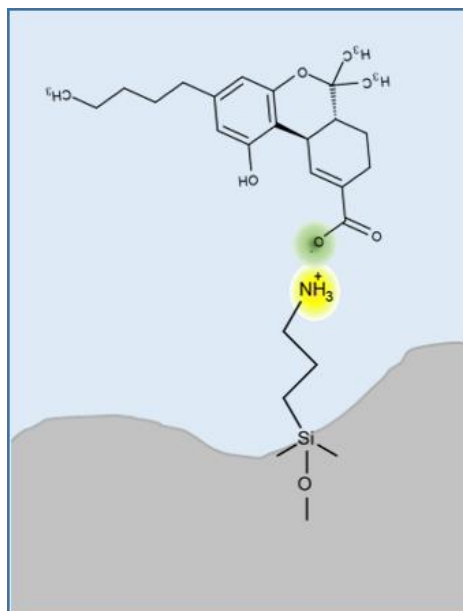


Figure 3-6 Mechanism of THC-COOH retention in the anion-exchange SPE columns

### 3.6.2 Experimental -SPE

After acidification of the basic hair digest solution, the steps described in Figure 3-7 was applied to the extraction of hair samples.

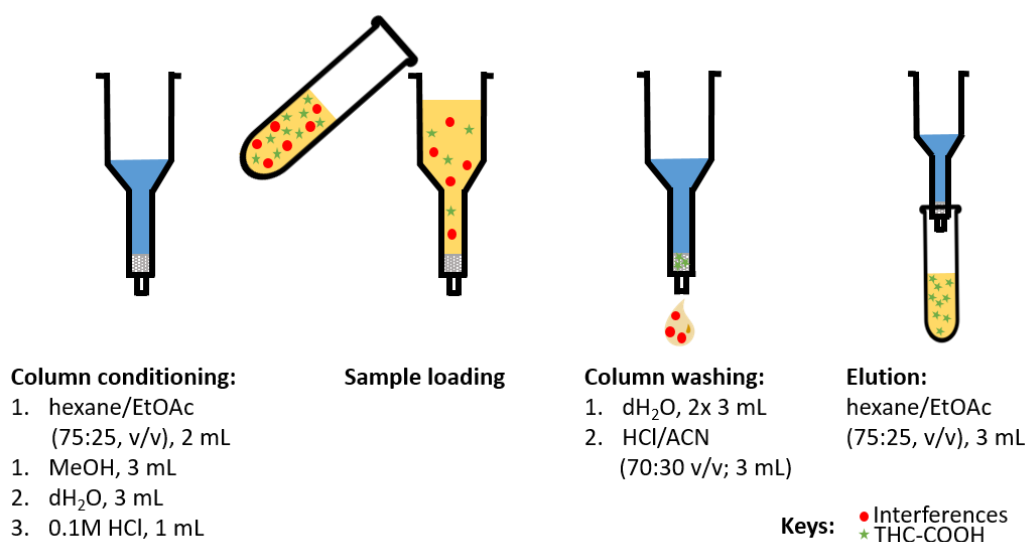
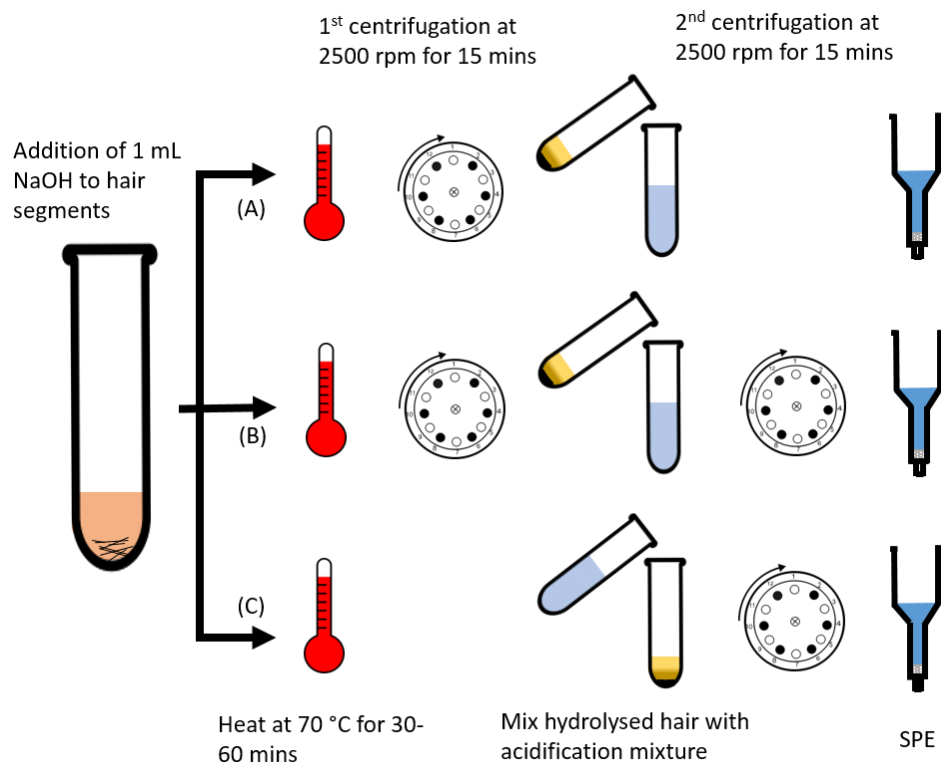


Figure 3-7 Schematic diagram of a solid-phase extraction procedure.

After hair digestion, as described in section 3.3.7, the solution was centrifuged for 10 mins at 2500 rpm. The supernatant was then poured into tubes containing acetic acid (1 mL), 1M acetic acid (3 mL), and 0.1M sodium acetate buffer (pH 4.5, 2 mL). The tubes were capped, vortex mixed for 30 seconds and loaded onto the already conditioned SPE column (Figure 3-8 'A'). However, as soon as the

acidification solution was added to the supernatant, the solution turned turbid and produced a foul smell which is due to the sulphur containing amino acid, cysteine which is present in the hair structure. The formed particulates were found to either completely impede the sample flow through the column, even under full vacuum; or, impractically, increase the sample flow time. Authors of the published work were contacted for consultation. The response received doubted the completeness of the hair digestion. Therefore, to roll out this possibility, incubation time of hair with NaOH was increased to 2 hours. However, this did not solve the problem. To overcome this problem two amendments were proposed as follow:

1. Method (B): addition of a 2<sup>nd</sup> centrifugation step after pouring the supernatant into the acidification mixture (Figure 3-8 'B').
2. Method (C): Elimination of the 1<sup>st</sup> centrifugation step, acidification of the sample and then centrifugation of the mixture prior to pouring the supernatant onto the extraction columns (Figure 3-8'C').



**Figure 3-8 Hair sample pre-treatment approaches prior to SPE.**

**(A) One centrifugation takes place after hair hydrolysis, prior to acidification, (B) Two centrifugations takes place after hair hydrolysis and after acidification, (C) One centrifugation takes place after acidification**



The effect of these changes on extraction recovery was then assessed by carrying out an extraction recovery study for both methods. Three sets of hair samples were prepared by weighing out 40-50 mg of hair. Each set consisted of two levels, 200 and 400 ng, analysed in triplicate.

### **3.7 Experimental - Drug loss monitoring**

Since only picogram/femtogram amounts of THC-COOH are incorporated into hair, any minor drug loss during sample preparation is significant. Recovery of THC-COOH from the standard derivatisation 3.5 mL vials was monitored. A calibration of eight concentrations (50, 100, 200, 500, 1, 2.5, 5 and 10 pg/50 mg) was derivatised by adding 30  $\mu$ L TFAA-HFIP and incubating in heating block at 80 °C for 30 minutes. The tube was then left to cool down at room temperature and then dried under a stream of nitrogen. Three sequential reconstitutions with 25  $\mu$ L toluene from each vial was transferred to labelled auto sampler vials and injected.

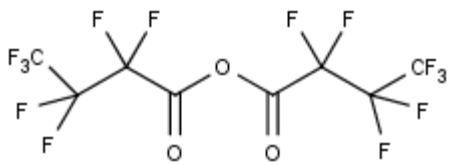
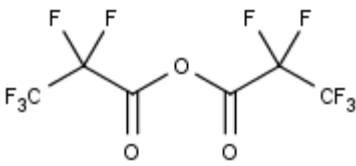
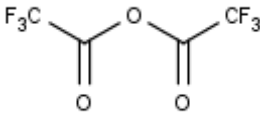
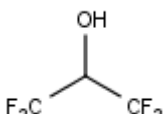
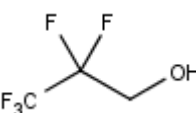
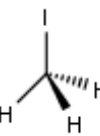
### **3.8 Derivatisation**

#### **3.8.1 Rationale for derivatisation**

Derivatization is a technique commonly employed in forensic toxicology to enhance chromatographic separation and mass spectrometric identification. It is used to make polar drugs and metabolites less polar and thermally stable for GC-MS analysis. This process increases the molecular weights of the molecule and hence improves chromatography and selectivity. The advantageous use of a derivatising agent could extend beyond the chromatography to the ionization efficiency and producing more characteristic mass spectra. For analysis of cannabinoids in hair, two complementary instruments are usually employed; standard GC-MS with electron ionisation (EI) and GC-MS with negative chemical ionisation (NCI). Detection of THC-COOH in hair matrices needs superior sensitivity due to its extremely low concentrations. For this purpose, an MS equipped with a NCI source is frequently reported in the literature. Introducing functional groups that contain more halogen atoms, which have high electron affinity (e.g. fluorine), is believed to improve the detectability of a compound with NCI by making it more electronegative (168). As THC-COOH contains free carboxylic acids as well as hydroxyl groups, combinations of derivatising reagents were used to introduce

fluorine atoms on both the carboxyl and hydroxyl groups and hence enhance ionisation efficiency and maximise sensitivity. In addition, the selective methylation of the carboxyl group is claimed to improve the sensitivity in a recently published work carried out using liquid chromatography- negative electrospray MS (LC-ESI-MS) (163). The carboxyl group on THC-COOH was esterified using methyl iodide, PFPOH, and HFIP, while the hydroxyl group was perfluorinated using PFPA, TFAA and HFBA. The chemical properties of derivatising agents employed are shown in Table 3-4.

**Table 3-4 The chemical Properties of the employed derivatisation reagents**

Reagent	Chemical structure	Chemical Formula	Molecular weight
HFBA		$C_6F_{14}O_3$	410.06
PFPA		$C_6F_{10}O_3$	310.05
TFAA		$C_4F_6O_3$	210.03
HFIP		$C_3H_2F_6O$	168.04
PFPOH		$C_3H_3F_5O$	150.05
Methyl iodide		$CH_3I$	141.94

### 3.8.2 Experimental

Initial testing of all derivatisation reagents was carried out using unextracted standards. 100  $\mu$ L of 1 $\mu$ g/mL individual working solutions with the same concentration of internal standard were transferred to 7 mL vials and dried under a stream of nitrogen before adding the derivatisation reagent. Derivatisation of

cannabinoids for GC-EI-MS analysis was achieved using 50 µl BSTFA with 1% TCMS. The vial was then capped and heated at 80 °C for 30 minutes.

For the purpose of comparing the derivatisation reagents for NCI, experimental work was carried out in three stages; firstly, determination of reaction conditions for all reagent combinations, secondly, assessment of the chromatographic behaviour and MS characteristics of all derivatives, thirdly, further comparison of sensitivity in SIM-MS mode for derivatives with satisfactory results.

#### **3.8.2.1 Conditions of derivatisation reactions**

According to the previous works in the literature, an incubation at 70-90 °C for 10-30 minutes would usually provide satisfactory reaction conditions for the following combinations; PFPOH-PFPA, PFPOH-TFAA, PFPOH-HFBA, HFIP-PFPA, HFIP-TFAA, HFIP-HFBA. Therefore, 30 µl of PFPOH and HFIP and 50 µL of TFAA, PFPA, and HFBA was added, each separately, to the reaction medium after drying the standard. The mixture was then incubated for 20 minutes at 80 °C. When the derivatisation reaction involved selective methylation of a carboxyl group, the reaction was carried out in two steps. First, alkylation of the carboxyl group while maintaining the phenolic hydroxyl group unchanged by addition of 100 µL acetonitrile, an excess of a few crystals of solid sodium carbonate and 20 µL methyl iodide. This mixture was incubated for 1.5 h at 70 °C. Secondly, to derivatise the hydroxyl group, the mixture was dried and either 50 µL of TFAA, PFPA, or HFBA was added and incubated for 20 minutes at 80 °C.

#### **3.8.2.2 Chromatographic behaviour and MS characteristics using full-scan mode**

Chromatographic separation and MS characteristics were assessed by derivatisation of 100 µl of THC-COOH working solution at 1µg/mL and an equivalent concentration of its deuterated ISTD THC-COOH-*d*3. Each derivative was injected separately using NCI mode with full-scan covering the range from 100-700 atomic mass units (amu).

#### **3.8.2.3 Sensitivity comparison of selected derivatives using SIM mode.**

Using GC-NCI-MS operated in SIM mode, two levels of unextracted standards at 100 and 1000 pg were derivatised and analysed in triplicate.

## 3.9 Results – LLE

### 3.9.1 THC, CBN, CBD, 11-OH-THC (Fraction 'A')

#### 3.9.1.1 Selection of extraction solvent

A range of solvents were assessed in order to determine which one extracted the most cannabinoids. The extract using DCM was excluded from the beginning as it was found to form white crystals that cannot be injected into the GC system. EtOAc and cyclohexane/EtOAc (9:1 v/v) were found to extract the most compounds. The mean percentage recoveries of THC, CBD, CBN and 11-OH-THC from digestion alkaline solution (1mL 1M NaOH) without hair using different solvents is shown in Table 3-5. All solvents achieved good recovery for THC ranging from 86.4% to 91.5%. MTBE gave good recoveries (>80.5%) with all analytes except CBD (mean=12.3%). The efficiency of pure cyclohexane for extracting all cannabinoids in fraction (a) simultaneously was found to be poor. Despite the good percentage recoveries of THC and, to lesser extent, CBD with cyclohexane, mean %recoveries 91.5 and 80.1, respectively, the percentage recoveries of the other two cannabinoids were much lower, 48.8% and 16.5%, for CBN and 11-OH-THC, respectively. Cyclohexane/EtOAc (9:1 v/v) and ethyl acetate were found to extract the most compounds with extraction recoveries ranging from 76.4% to 96.6%. Therefore, an extraction recovery of spiked blank hair matrices was carried out using cyclohexane/EtOAc (9:1 v/v) and ethyl acetate. Despite the fact that the percentage recoveries of most analytes dropped by 10-30%, both solvents gave reasonable percentage recoveries with the exception of CBD as shown in Table 3-6. It should, however, be noted that the solvent mixture cyclohexane/EtOAc, of high non-polar solvent content, gave a relatively clean extract.

**Table 3-5 Mean extraction recoveries of cannabinoids from 1 mL 1M NaOH without hair**

Solvent	THC	CBD	CBN	11-OH-THC
Cyclohexane/EtOAc (9:1 v/v)	89.3	96.6	76.4	78.9
Cyclohexane	91.5	80.1	48.8	16.5
EtOAc	91.3	94.9	76.7	90.8
MTBE	86.4	12.3	80.5	88.1

**Table 3-6 Mean extraction recoveries of cannabinoids from 50 mg of hair digested with 1 mL 1M NaOH using the solvent that achieved the recovery without hair.**

Solvent	THC	CBD	CBN	11-OH-THC
Cyclohexane/EtOAc (9:1 v/v)	70.3	28.7	53.6	68.6
EtOAc	82.1	14.8	38.3	85.9

### 3.9.1.2 Optimisation of solvent composition

Although 9 to 1 solvent composition of cyclohexane to EtOAc was found to be the best in the previous experiment, the percentage recoveries of CBD, CBN and 11-OH-THC were found to vary significantly between days. Cyclohexane/EtOAc (3:1, v/v) instead of (9:1, v/v) was proposed and assessed. This new solvent composition (3:1, v/v) gave a better extraction percentage recovery for 11-OH-THC. The other cannabinoids, THC, CBN and CBD, gave similar percentage recoveries at the two solvent compositions as shown in Table 3-7

**Table 3-7 Mean extraction recoveries of cannabinoids from hair digested with 1 mL NaOH using cyclohexane/EtOAc mixture with two different compositions.**

Solvent	THC	CBD	CBN	OH-THC
Cyclohexane/EtOAc (9:1 v/v)	86.4	105.1	107.5	37.6
Cyclohexane/EtOAc (3:1 v/v)	97.2	95.9	94.2	87.9

### 3.9.1.3 Effect of centrifuge duration

Centrifuge duration was assessed at 10 and 30 minutes. The results of extraction recovery and a visual examination of the chromatogram shows that centrifuge duration does not have a significant effect on the extraction yield or cleanliness of the extract. Extraction recoveries were similar to the values reported previously for Cyclohexane/EtOAc (3:1 v/v) in Table 3-7.

## 3.9.2 THC-COOH (Fraction 'B')

Extraction recovery results with different acidification mixtures are shown in Table 3-8. Acetic acid seems to be the most appropriate acidification method. The lowest volume of acetic acid (120 µl) resulted in the best recovery with good reproducibility. It was not possible to calculate the percentage recovery of THC-COOH from after acidification using the other three methods. No response for THC-COOH was recorded.

**Table 3-8 Mean extraction recoveries of THC-COOH from hair digested with 1 mL NaOH using cyclohexane/EtOAc mixture with two different acidification methods.**

Acidification method	THCA	s	CV
120 µl Acetic acid	71.8	4.5	6.2
500 µl Acetic acid	65.3	6.6	10.1
1000 µl Acetic acid	61.2	10.8	17.7

### 3.9.3 Simultaneous extraction of all cannabinoids using LLE

The early acidification of hair digest with 100 µl HCl resulted in acceptable recovery for THC and 11-OH-THC and poor recovery for THC-COOH as shown in Table 3-9. It was not possible to calculate the percentage recovery of CBD and CBN as no peaks were recorded for the monitored ions.

**Table 3-9 Mean extraction recoveries of cannabinoids from hair digested with 1 mL NaOH and acidified with 100 µl HCl prior to LLE with cyclohexane/EtOAc mixture.**

	THC	11-OH-THC	THC-COOH
mean	86.74	80.41	14.76
s	9.34	7.77	2.23
cv	10.77	9.66	15.13

### 3.10 LLE versus SALL for THC-COOH

Table 3-10 shows the percentage extraction recoveries of THC-COOH using LLE and SALL. It can be clearly concluded that LLE provided a better recovery. No significant improvement in extract cleanliness was noticed. Therefore, SPE method was tested.

**Table 3-10 Mean, standard deviation and coefficient of variation of THC-COOH percentage recovery using LLE and SALL**

	mean	s	CV
LLE	98.16	16.22	16.52
SALL	57.23	6.99	12.21

### 3.11 SPE for THC-COOH

Table 3-11 shows the percentage extraction recoveries of THC-COOH from hair matrices using SPE. Three different approaches were compared. Despite the superior recovery that was noticed with approach (A), it was excluded due to the

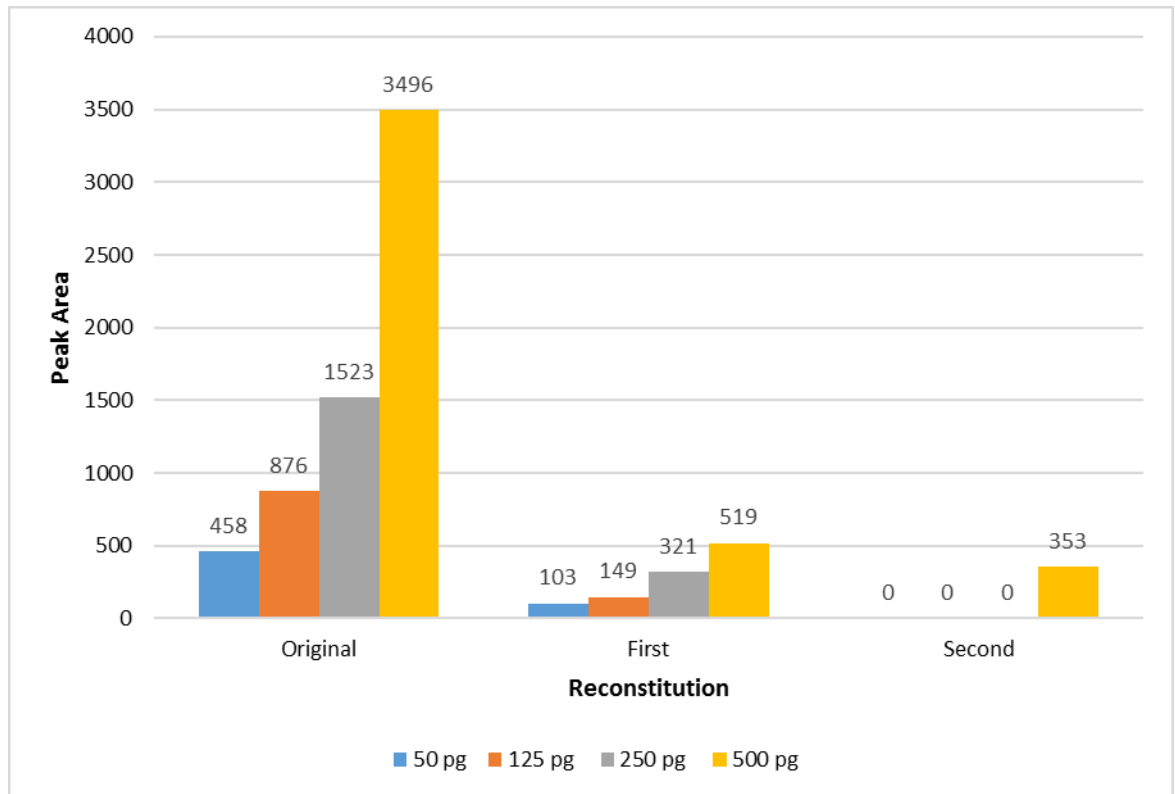
reasons discussed earlier is section 3.11.2. Approaches (B) and (C) were found to have similar extraction efficiencies with slight superiority for approach (C). Therefore, approach (C), which involves one centrifugation step conducted after sample acidification, was employed for method validation and case sample analysis.

**Table 3-11 Mean, standard deviation and coefficient of variation of THC-COOH percentage recovery using SPE after three approaches for hair sample pre-treatment**

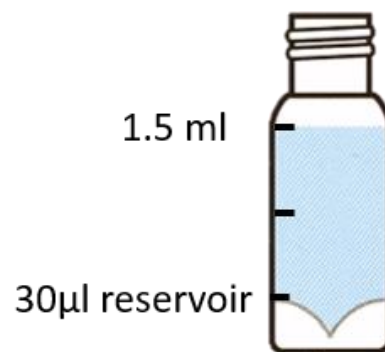
	Mean	s	CV
Approach (A)	58.4	10.8	18.5
Approach (B)	40.3	18.1	44.9
Approach (C)	48.9	21.1	43.1

### 3.12 Drug loss monitoring

As shown in Figure 3-9, the first reconstitution from the 50, 125, 250 and 500 pg vials and second reconstitution of the highest level were found to show a response which means some of the drug was left over in the reaction medium. The signal from the first reconstitution was calculated and ranged from 14.9% to 22.4% of the original signal. To improve detection sensitivity, the step of transferring the organic layer to a 7 mL vial for derivatisation and then to an auto sampler vial for injection was shortened. Instead, the LLE organic layer was transferred straight into a silanised high recovery vial (See Figure 3-10) which was used as a derivatisation medium and an auto-sampler vial for injection.



**Figure 3-9 Comparison of detector response for the original, first and second reconstitutions of 4 levels of unextracted standards**



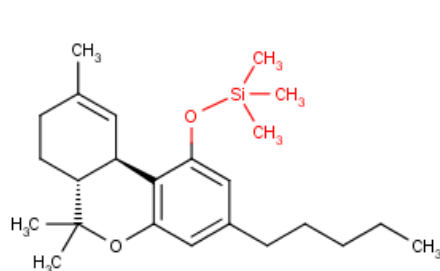
**Figure 3-10 Silanised High Recovery (HR) vial**



### 3.13 Results – Derivatisation

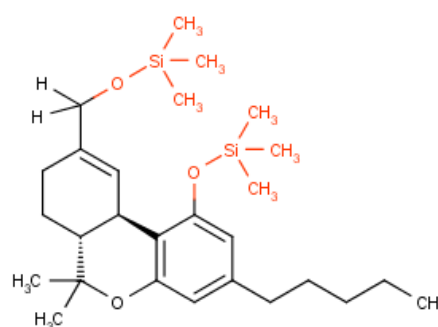
#### 3.13.1 Derivatisation of THC, CBD, CBN, 11-OH-THC and THC-COOH for GC-EI-MS analysis

Derivatisation with trimethylsilyl (TMS) is most commonly used when GC-MS with EI detection is the employed analytical technique (**system 1**). TMS derivatives are formed by attaching to the hydroxyl and/or carboxyl groups present on the THC, CBD, CBN, 11-OH-THC, and THC-COOH structures. Structures of formed cannabinoids TMS derivatives are shown in Figure 3-11.



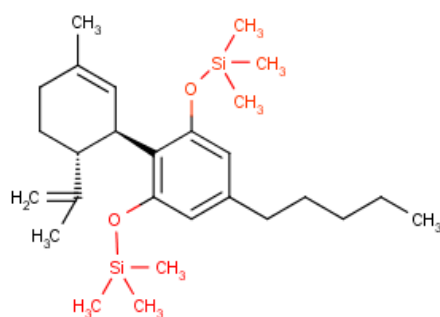
THC-TMS

MW = 386



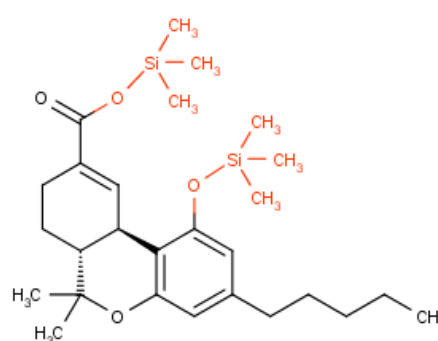
11-OH-THC-2-TMS

MW = 474



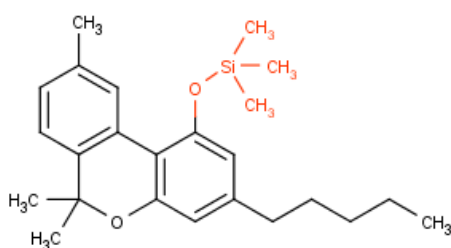
CBD-2-TMS

MW = 458



THC-COOH-2-TMS

MW = 488



CBN-TMS

MW = 382

Figure 3-11 Chemical structure of cannabinoids TMS derivatives

The extracted ion chromatograms and full scan electron ionisation mass spectra for THC, CBD, CBN, 11-OH-THC, THC-COOH, their deuterated ISTD THC-*d*3, 11-OH-THC-*d*3 and THC-COOH-*d*3 and standard TMS derivatives are shown in Figure 3-12 to Figure 3-19.

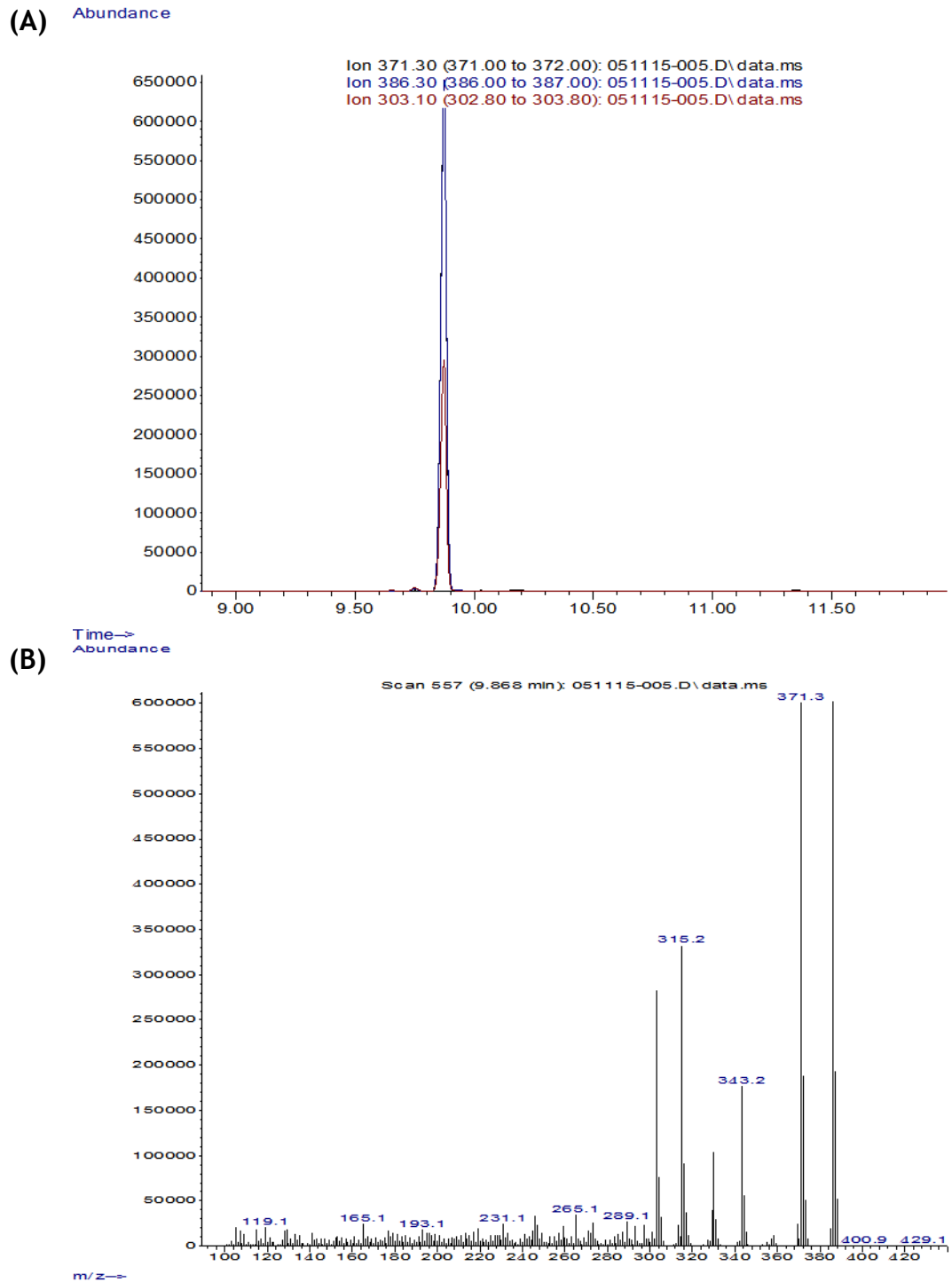
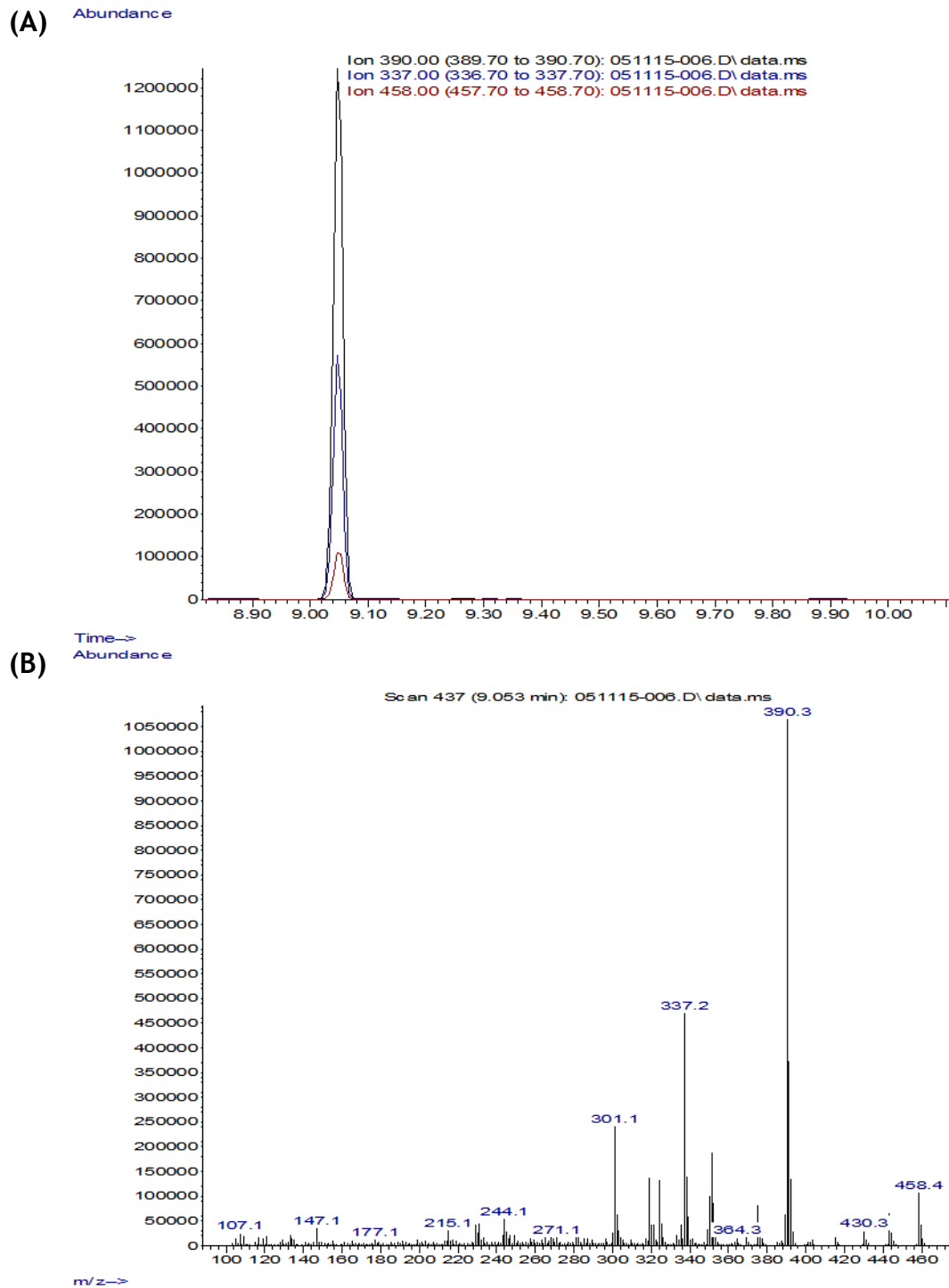


Figure 3-12 Extracted ion chromatograms for THC-TMS [ $m/z$  371, 386, 303] (A), and its full scan electron ionisation (EI) mass spectra (B).



**Figure 3-13** Extracted ion chromatograms for CBD-2-TMS [ $m/z$  390, 337, 458] (A), and its full scan electron ionisation (EI) mass spectra (B).

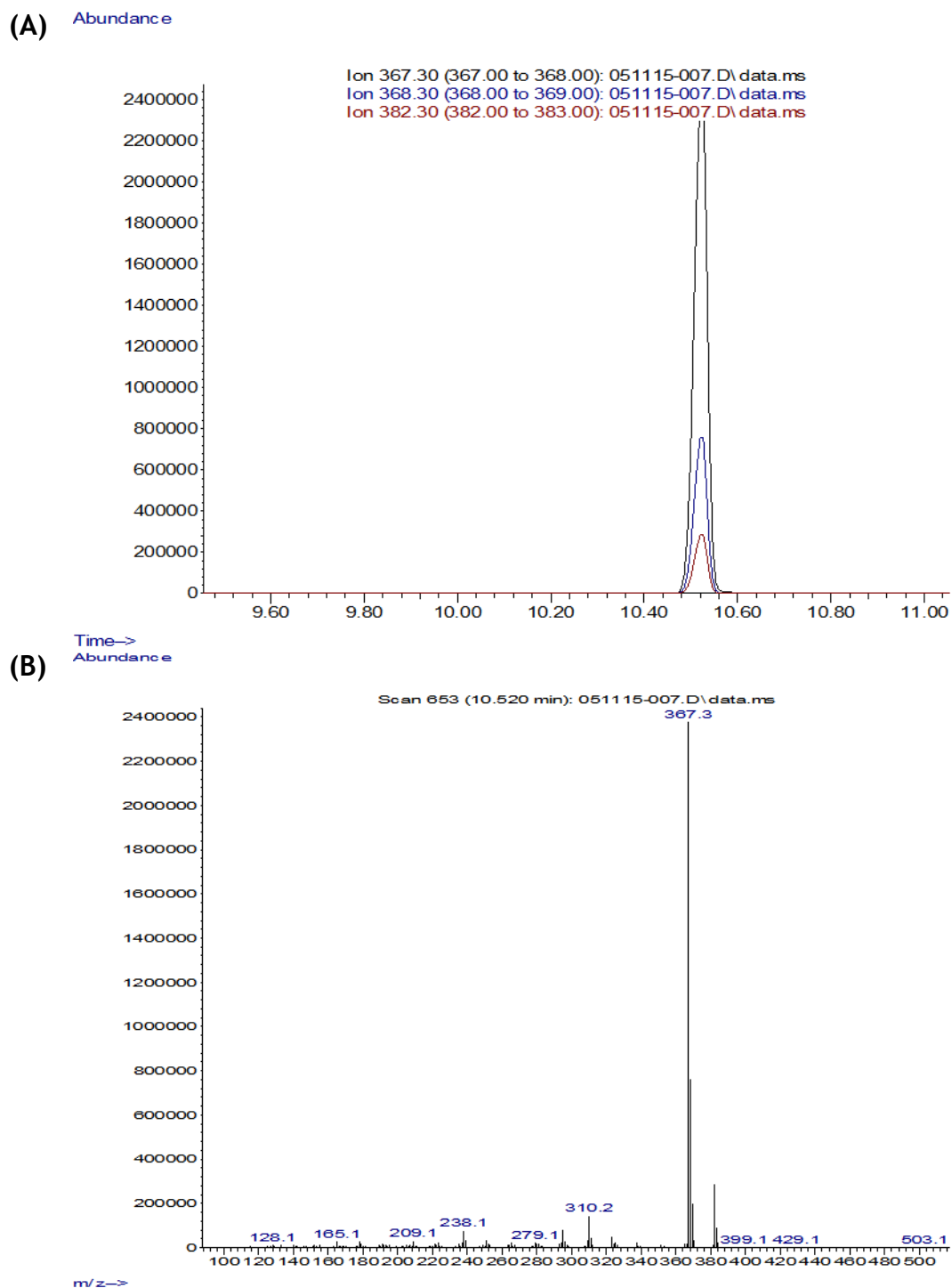
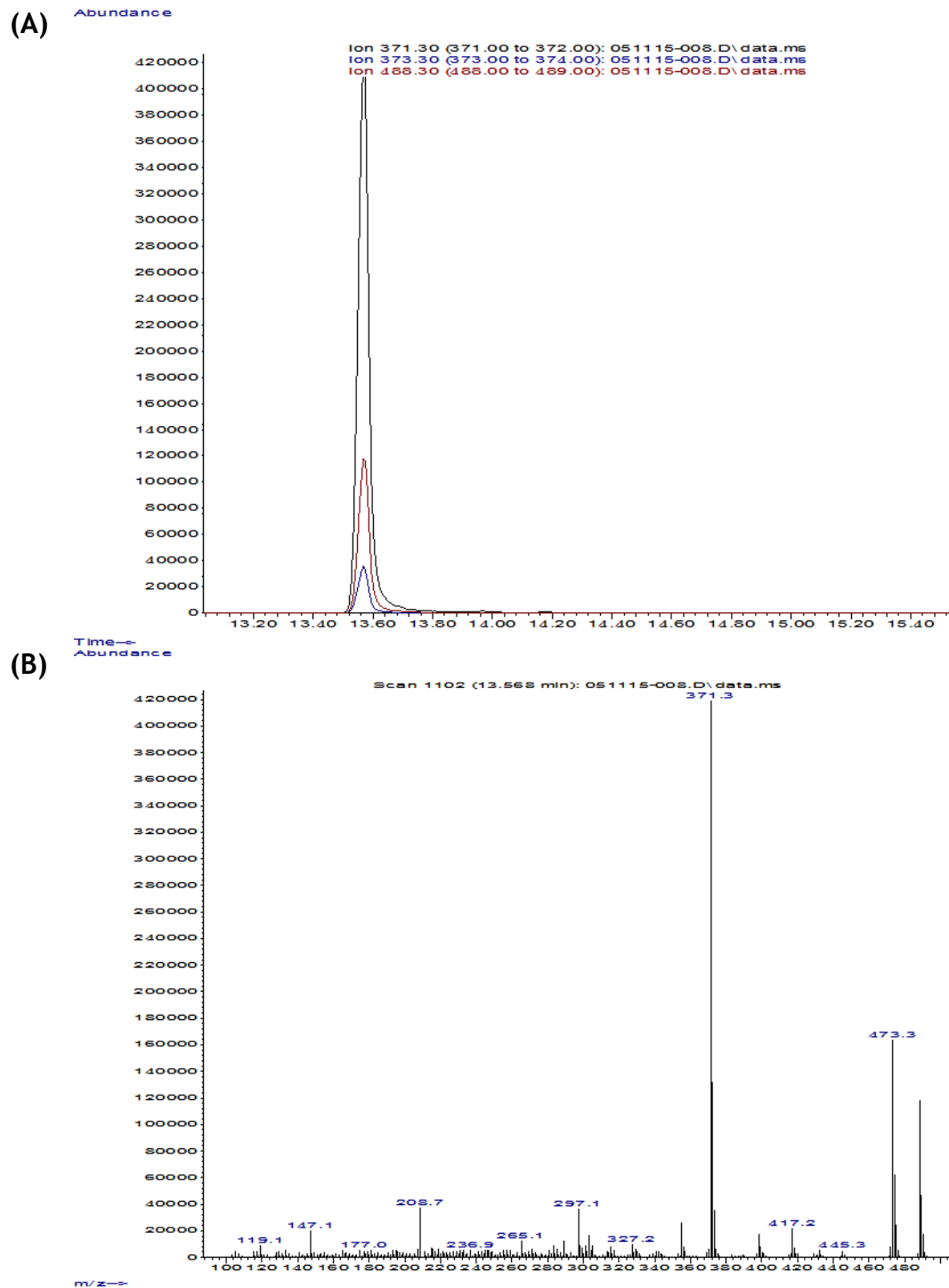
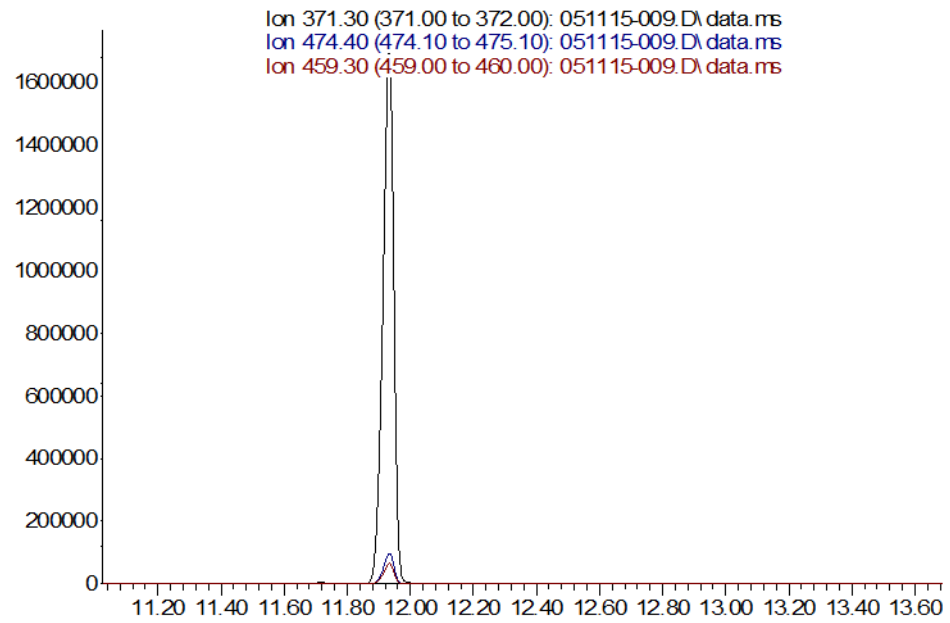


Figure 3-14 Extracted ion chromatograms for CBN-TMS [ $m/z$  367, 368, 382] and its full scan electron ionisation (EI) mass spectra (B).



**Figure 3-15** Extracted ion chromatograms for THC-COOH-2-TMS [ $m/z$  371, 373, 488] (A), and its full scan electron ionisation (EI) mass spectra (B).

(A) Abundance



(B) Time→  
Abundance

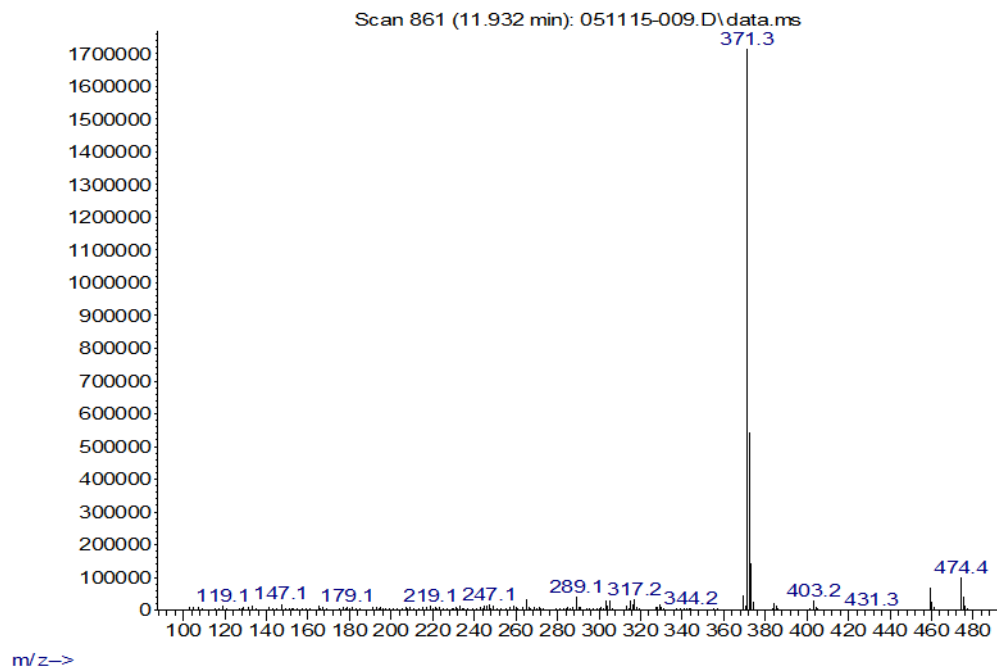


Figure 3-16 Extracted ion chromatograms for 11-OH-THC-2-TMS [ $m/z$  371, 474, 459] (A), and its full scan electron ionisation (EI) mass spectra (B).

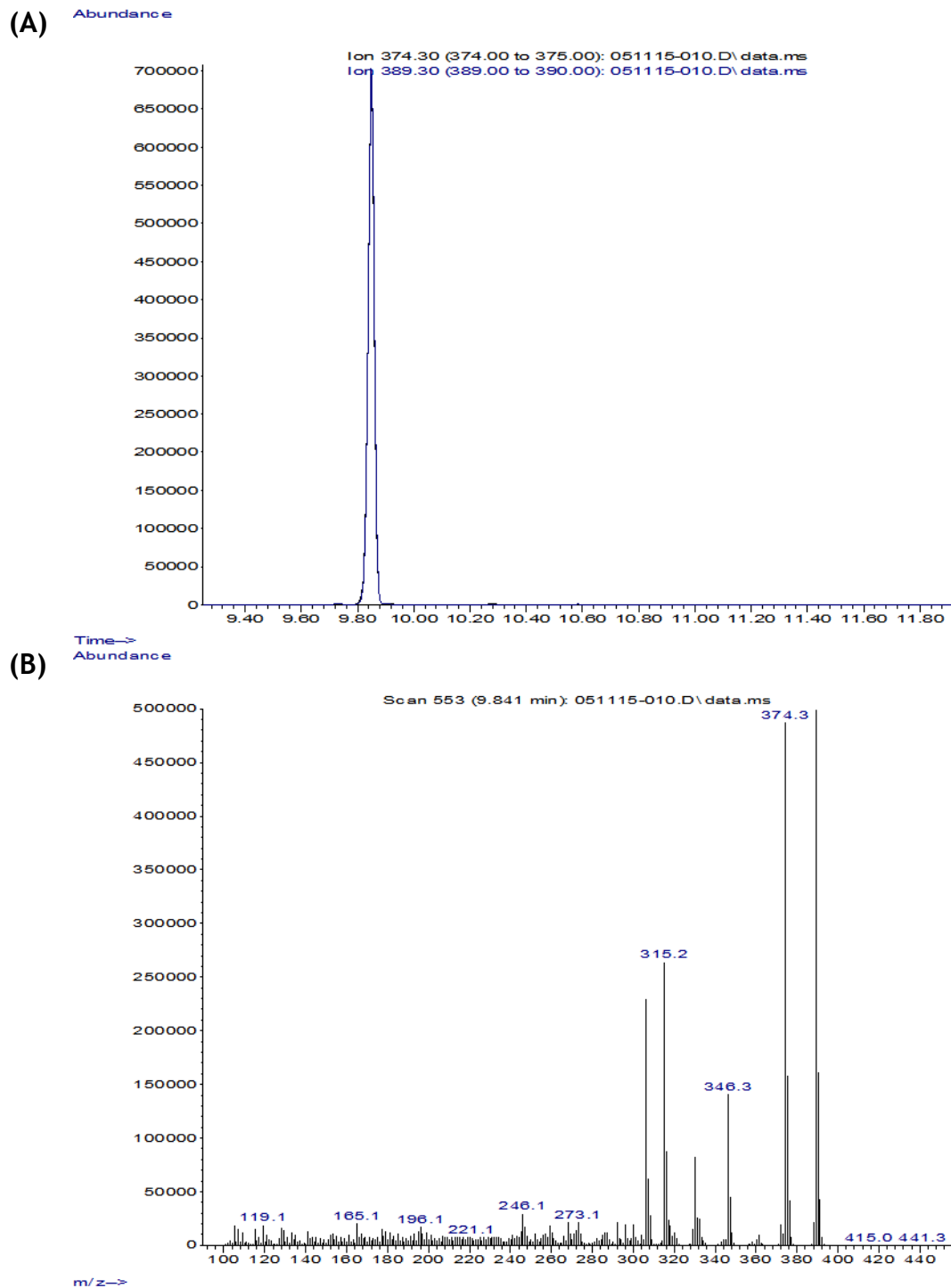


Figure 3-17 Extracted ion chromatograms for THC-*d*3-TMS [*m/z* 374, 389] (A), and its full scan electron ionisation (EI) mass spectra (B).

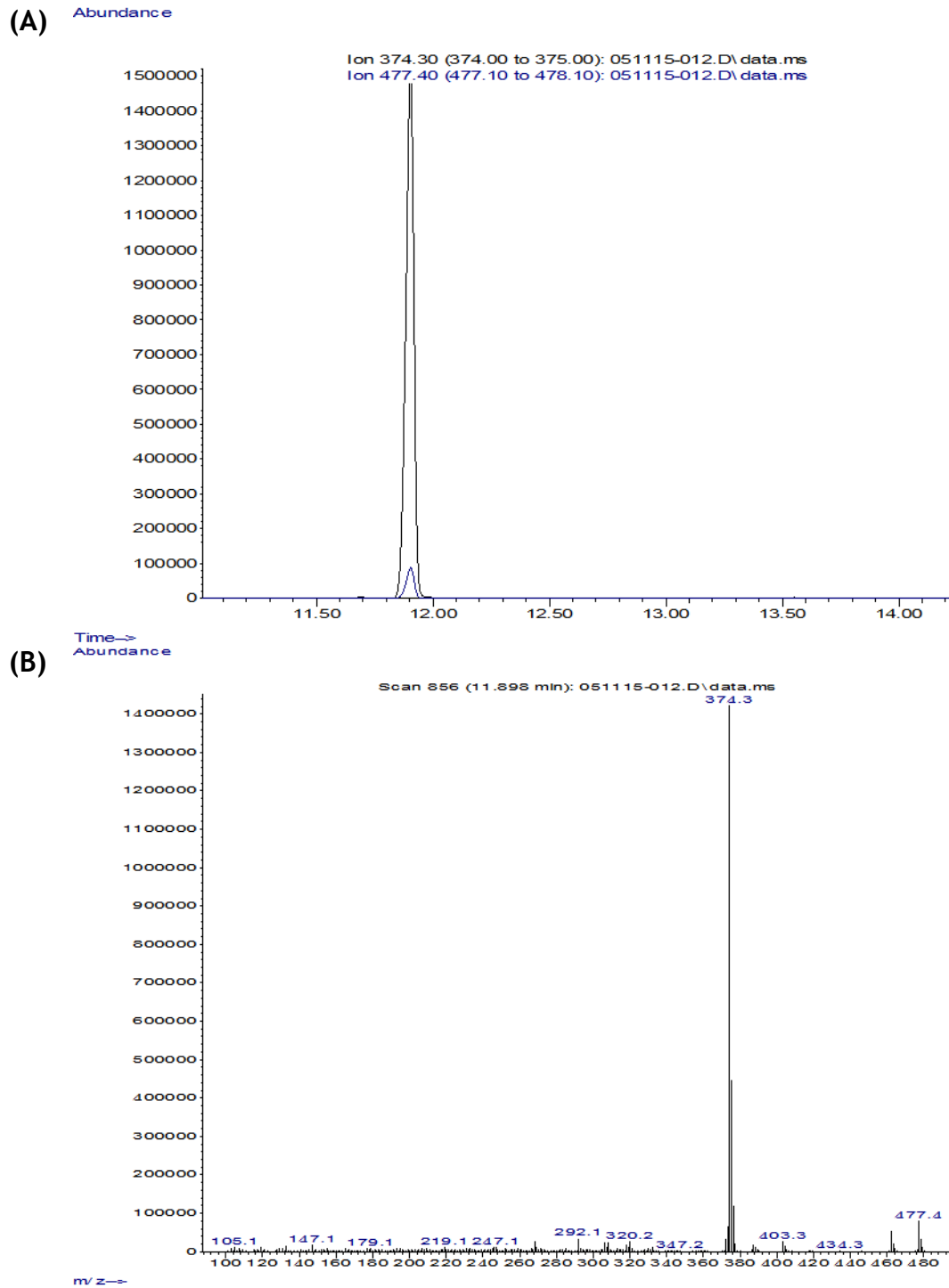


Figure 3-18 Extracted ion chromatograms for 11-OH-THC-*d*3-2-TMS [*m/z* 374, 477] (A), and its full scan electron ionisation (EI) mass spectra (B).



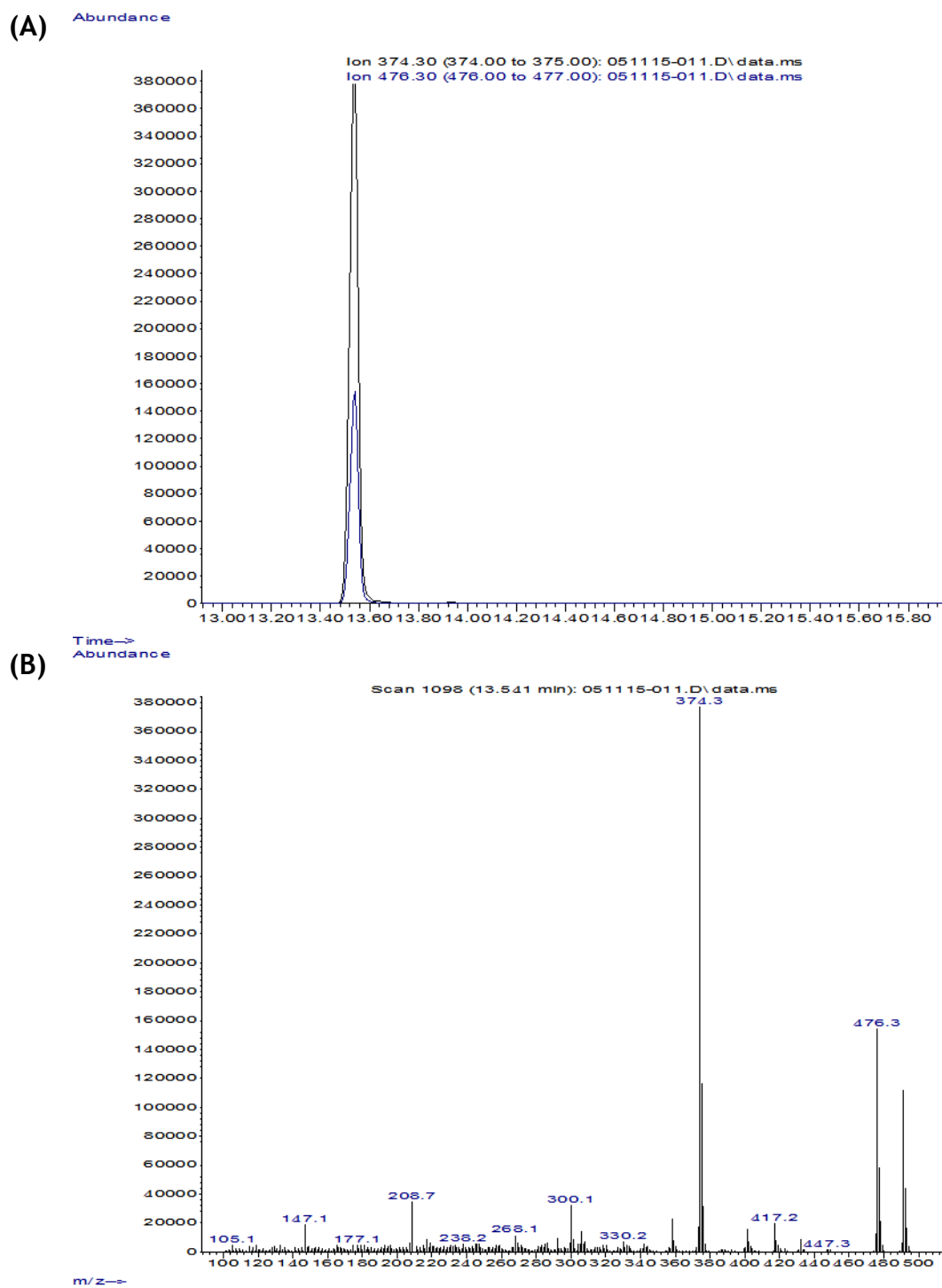


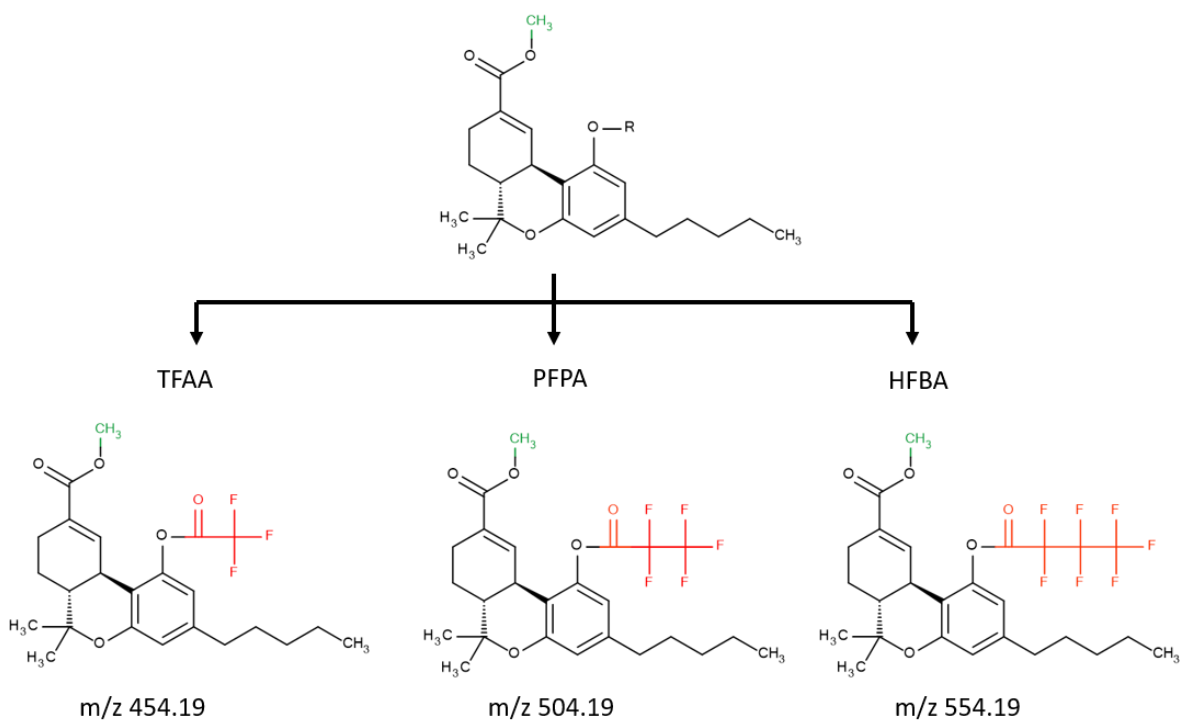
Figure 3-19 Extracted ion chromatograms for THC-COOH-*d*3-2-TMS [*m/z* 374, 476] (A), and its full scan electron ionisation (EI) mass spectra (B).

THC and CBD have the same molecular mass prior to derivatisation. As there are two hydroxyl groups on the CBD structure and only one hydroxyl group present on the THC structure, different retention times and mass spectra are elucidated after derivatisation with silylation reagent. This is not achievable with some other derivatisation reagents such as perfluorinated anhydrides. The use of perfluorinated anhydrides alone or coupled with perfluoroalcohols, such as HFIP/TFAA or PFPOH/PFPA, will result in identical retention times and mass spectra. If analysis of one of the two only is of interest, consideration must be given to the possibility of the presence of the other in a sample when choosing the method of derivatization. Otherwise, a false positive or high concentration will be reported for THC.

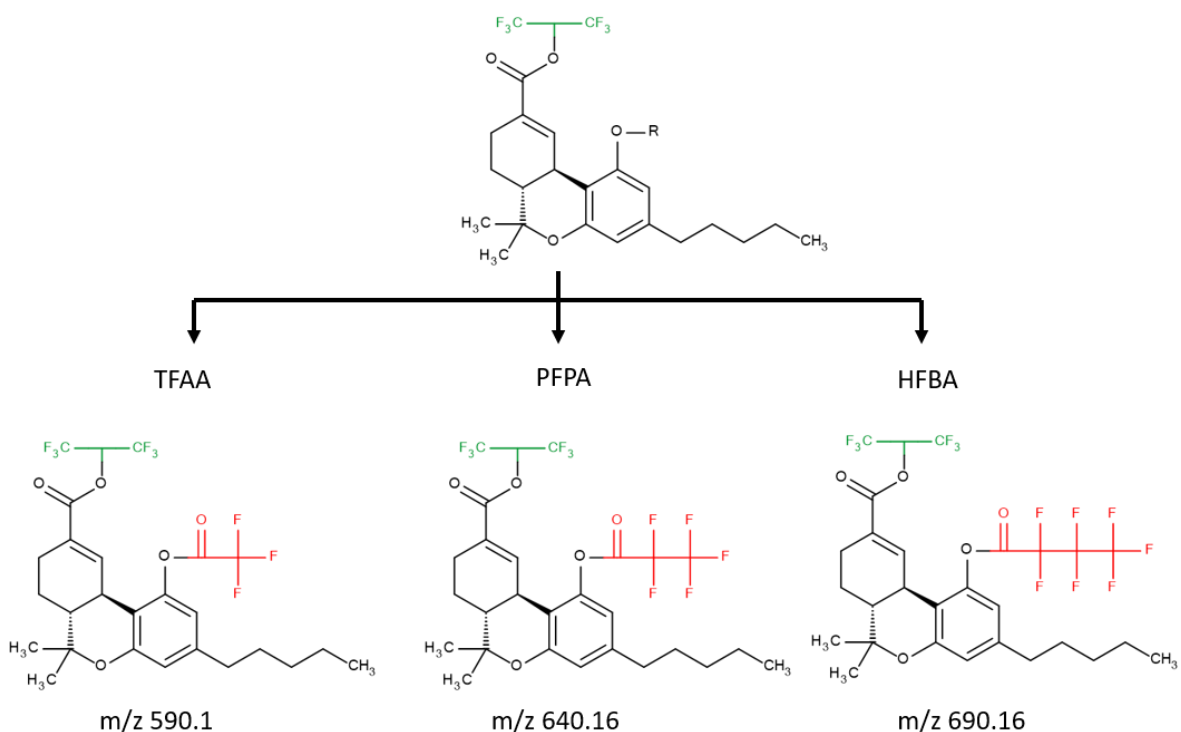
### **3.13.2 Derivatisation of THC-COOH for 2D GC-NCI-MS analysis**

#### **3.13.2.1 Conditions of derivatisation reactions**

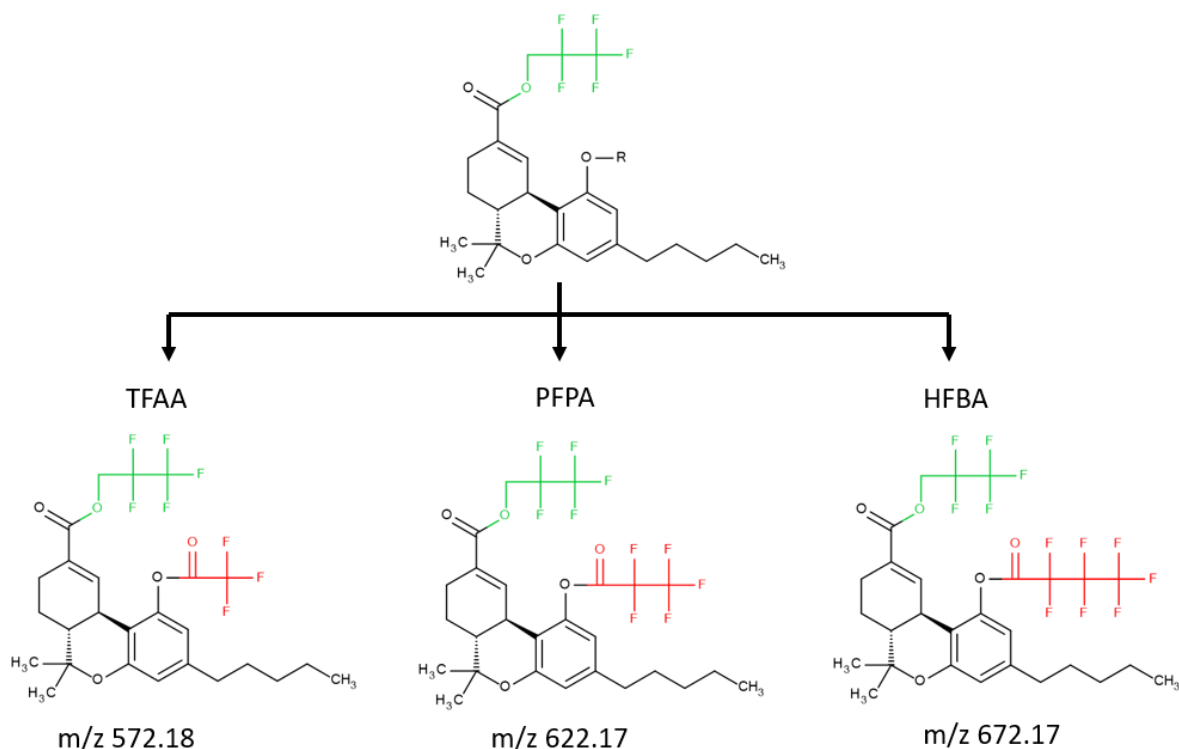
Determination of optimal reaction conditions for all reagent combinations was achieved by reviewing the literature for frequently reported conditions. As the primary aim was to select the combination that provides satisfactory chromatography and mass spectra characteristics, no further optimisation was carried out. Chemical structures of all combinations are shown in Figure 3-20, Figure 3-21 and Figure 3-22.



**Figure 3-20 Chemical structures of THC-COOH derivatives by esterification of carboxyl group with iodomethane and acylation of hydroxyl group with three perfluorinated anhydrides; TFAA, PFPA, and HFBA.**



**Figure 3-21 Chemical structures of THC-COOH derivatives by esterification of carboxyl group with HFIP and acylation of hydroxyl group with three perfluorinated anhydrides; TFAA, PFPA, and HFBA.**



**Figure 3-22 Chemical structures of THC-COOH derivatives by esterification of carboxyl group with PFPOH and acylation of hydroxyl group with three perfluorinated anhydrides; TFAA, PFPA, and HFBA.**

### 3.13.2.2 Chromatographic behaviour and MS characteristics using full-scan mode

Analysis of derivatives was carried out using 2D GC-MS in full-scan mode. As unextracted standards were used, the deans switch valve was kept on all the time and the cryo focusing trap was switched off with all derivatives to produce comparable data for all combinations. Therefore, there was no need to identify the retention time for each derivative on the FID at this stage. Ammonia was employed as buffer gas in NCI due to its superior thermalizing power. This power is estimated to be approximately seven times higher than methane. However, less fragmentation is expected with ammonia in the chemical ionization spectrum. This is because the proton affinity of ammonia (204.0 kcal/mole) is higher than that of methane; hence, less energy is transferred in the ionization reaction.

Chromatographic and mass spectrometric identification data were assessed according to the European Commission (EC) recommendations (169). The minimum acceptable retention time for the analyte under examination is twice the retention time corresponding to the void volume of the column. All derivatives were found to elute from the two capillary columns at acceptable retention times.

The shortest retention time was achieved with the HFIP-PFPA derivative at 9.8 minutes. Chromatographic retention times and MS characteristics of all derivatives are shown in Figure 3-23, Figure 3-24, and Figure 3-25, and summarised in Table 3-12. All derivatives that involve methylation of carboxyl group with iodomethane were found to have poor mass spectra with only one ion of good intensity. The best mass spectra were produced from derivatives that involved using HFIP for esterification of the carboxyl group. Therefore, these three derivatives were subject to further comparison of their sensitivity using SIM mode.

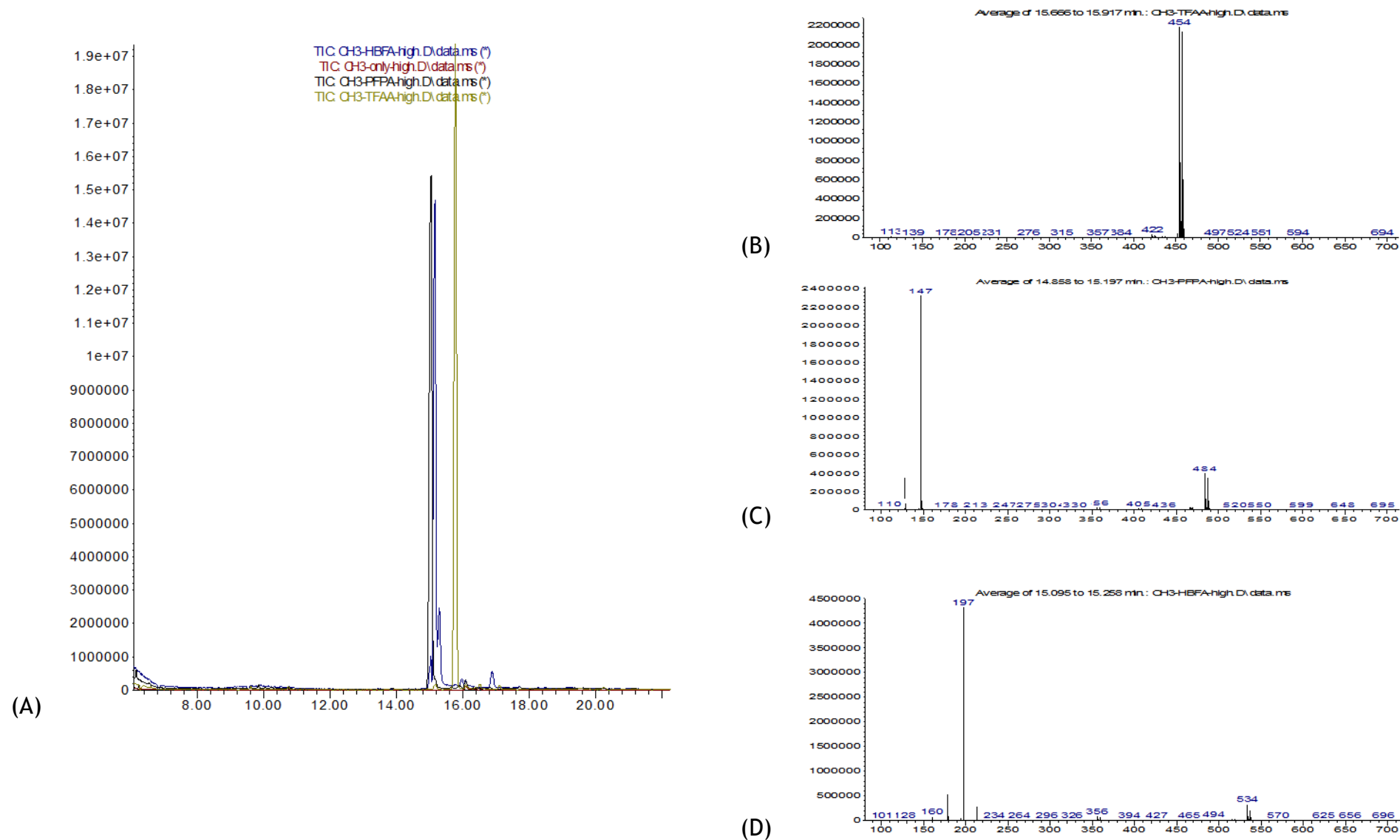


Figure 3-23 FS chromatograms of THC-COOH derivatives using a unextracted standard at 1 $\mu$ g/mL, (B) FS-NCI-MS of THC-COOH- iodomethane-TFAA, (C) FS-NCI-MS of THC-COOH- iodomethane -PFPA, (D) FS-NCI-MS of THC-COOH- iodomethane -HBFA derivative

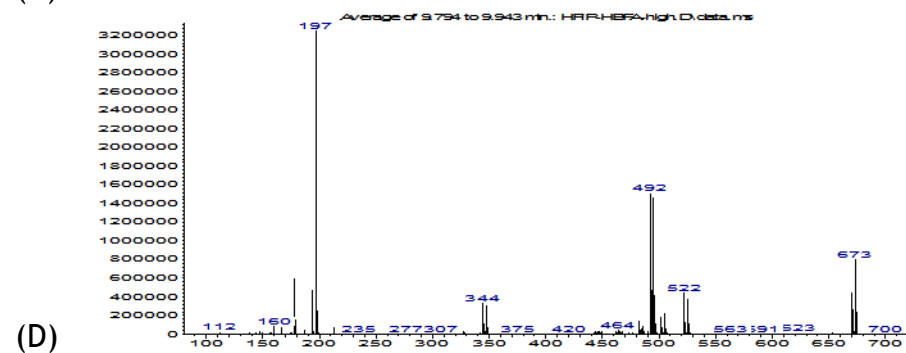
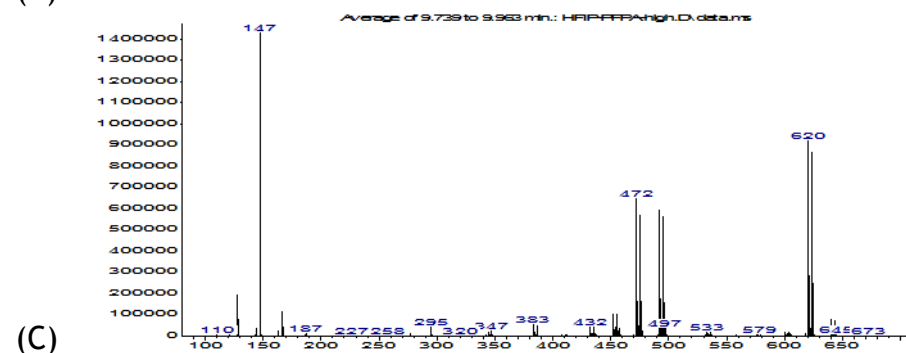
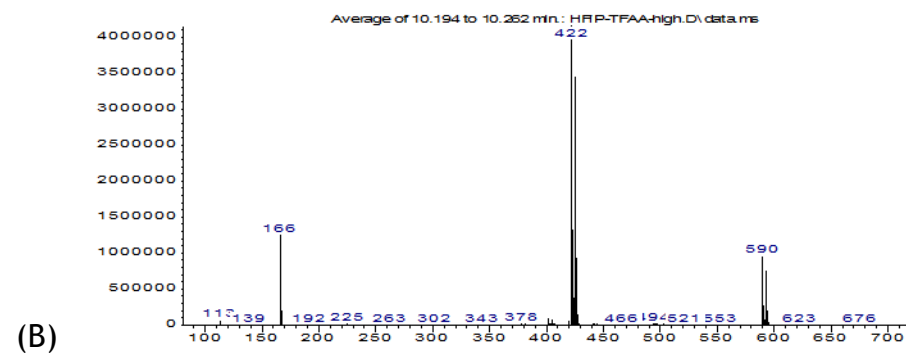
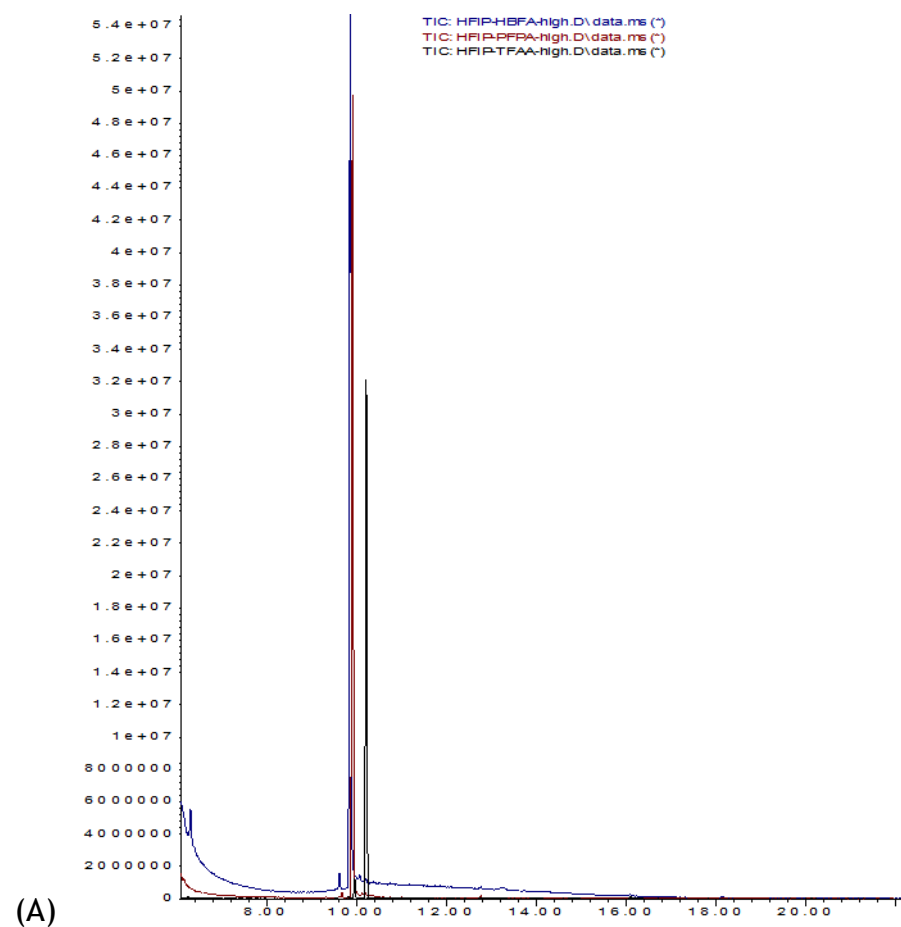


Figure 3-24 FS chromatograms of THC-COOH and THC-COOH-*d*3 derivatives using a unextracted standards at 1 µg/mL, (B) FS-NCI-MS of THC-COOH-HFIP-TFAA, (C) FS-NCI-MS of THC-COOH-HFIP-PFPA, (D) FS-NCI-MS of THC-COOH-HFIP-HBFA derivative

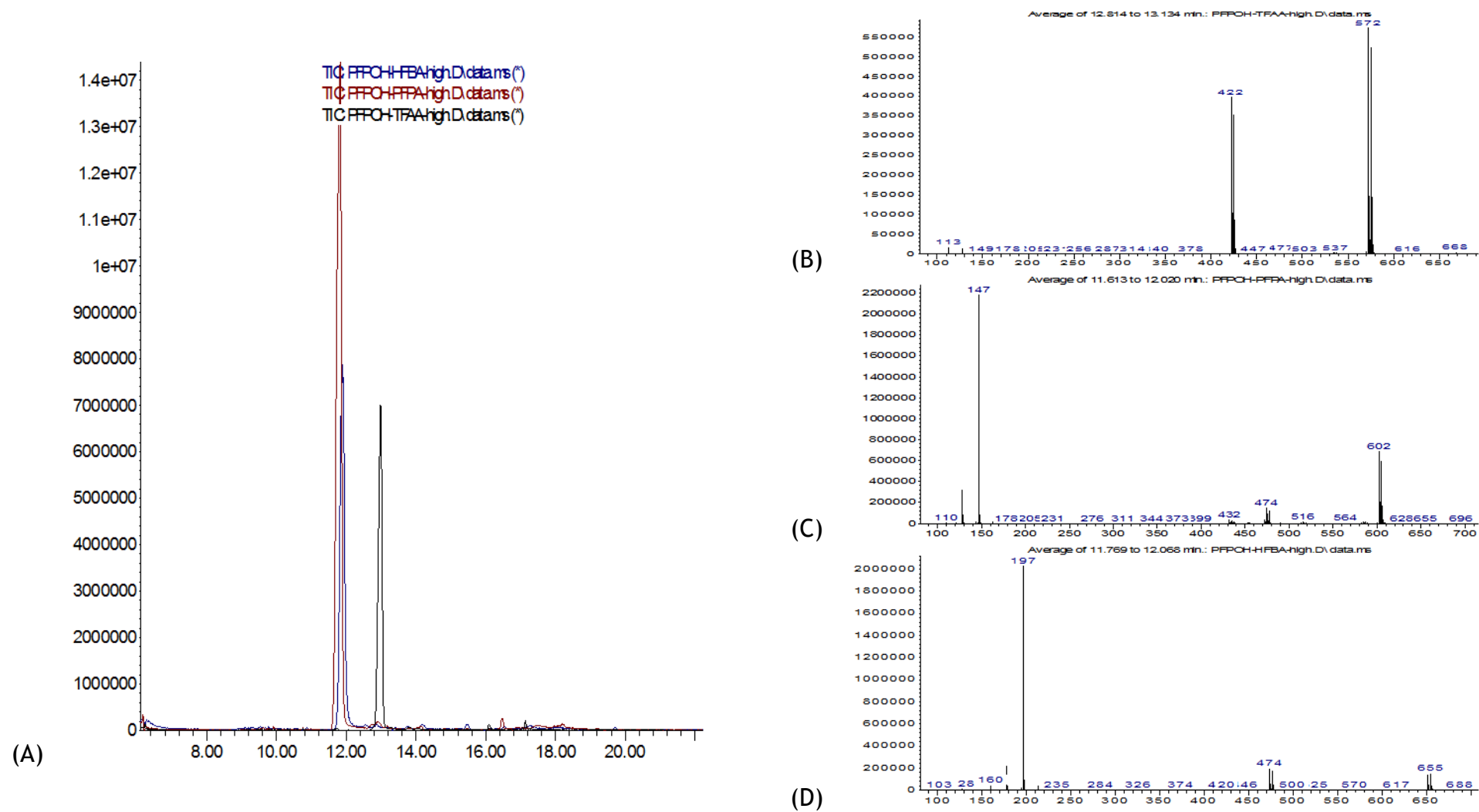


Figure 3-25 (A) FS chromatograms of THC-COOH derivatives using a unextracted standard at 1 µg/mL, (B) FS-NCI-MS of THC-COOH-PFPOH-TFAA, (C) FS-NCI-MS of THC-COOH-PFPOH-PFPA, (D) FS-NCI-MS of THC-COOH-PFPOH-HFBA derivative .....



Table 3-12 Summary of derivatisation combinations RT and fragmentation ions.

Combination	RT (min)	drug ions (m/z)	IS ions (m/z)	Common
CH <sub>3</sub> -TFAA	15.8	<u>454</u>	457	-
CH <sub>3</sub> -PFPA	15.0	<u>484</u>	487	147
CH <sub>3</sub> -HFBA	15.2	<u>534</u>	537	197
HFIP-TFAA	10.2	<u>422</u> , 590	425, 593	166
HFIP-PFPA	9.8	<u>620</u> , 472, 492	623, 475, 495	147
HFIP-HFBA	9.9	<u>492</u> , 670, 522, 344	495, 673, 525, 347	197
PFPOH-TFAA	13.0	<u>572</u> , 422	575, 425	-
PFPOH-PFPA	11.8	<u>602</u> , 474	605, 477	147
PFPOH-HFBA	11.9	<u>652</u> , 474	655, 477	197

Underlined ions are for fragments with highest intensities.

### 3.13.2.3 Sensitivity comparison of selected derivatives using SIM mode.

In SIM mode, the instrument is set to gather data at the masses of interest, instead of stepping the mass filter over a wide range of masses. Since the mass spectrometer collects data at only the masses of interest, it responds only to those compounds that possess the selected mass fragments. In essence, the instrument is focused on only the compounds of interest. Also, because only a few masses are monitored, much more time may be spent looking at these masses, with the attendant increase in sensitivity. Ions of highest intensity were chosen for SIM methods. Ions that were found mutual in the analyte of interest and its deuterated ISTD were avoided. European Commission recommends a minimum of 3 identification points for confirmation assays of cannabinoids using GC-El or CI-MS. Each ion in SIM mode is counts as one point. Therefore, three ions are necessary for satisfactory compound identification. It was possible to select three ions for HFIP-PFPA and HFIP-HFBA derivatives, however, only two ions were available from the HFIP-TFAA derivative mass spectrum for the SIM method. In addition to the analyte ions, two ions from the deuterated ISTD derivatives were added to each SIM group. Due to the very close retention times of the derivatives, three separate SIM methods were created for analysis. The methods shared exactly the same parameters except MS ions and dwell time. Dwell time for each SIM group was ensured to yield 15-20 cycles across a peak. Therefore, 100 ms was selected with

4 ions per group, and 50 ms for 5 ions per group. After triplicate analysis using unextracted standards (100 and 1000 pg), the signals produced from HFIP-PFPA and HFIP-HFBA derivative ions were slightly better than those from TFAA-HFIP. The combination of HFIP-PFPA was initially selected as the derivatisation reagent. However, the reaction outcome was not reproducible when used for derivatisation of the hair extract later in the study. The best derivatisation combination that produced acceptable sensitivity and reproducible data was determined to be TFAA-HFIP.

### 3.14 Summary

The work carried out in this chapter was to select the optimal extraction conditions for all cannabinoids from hair samples. Cyclohexane:EtOAc (3/1, v/v) was found to be the best extracting solvent for THC, CBD, CBN and 11-OH-THC. LLE resulted in better extraction recoveries for THC, CBD, CBN and 11-OH-THC from hair than SALL. SPE was selected as the best option for extraction of THC-COOH from hair. Acidification of the hair digest followed by centrifugation was found to be the most practical option for preparing the hair digest for SPE. Silanised high recovery vials were employed with both extractions and resulted in at least 15% improvement in detection sensitivity. Derivatisation of THC-COOH with TFAA-HFIP combination was the most appropriate option that provided reproducible reaction outcomes.

## Chapter 4 **Method development and validation for cannabinoids using GC-EI-MS and 2D GC-NCI-MS**

### 4.1 **Introduction**

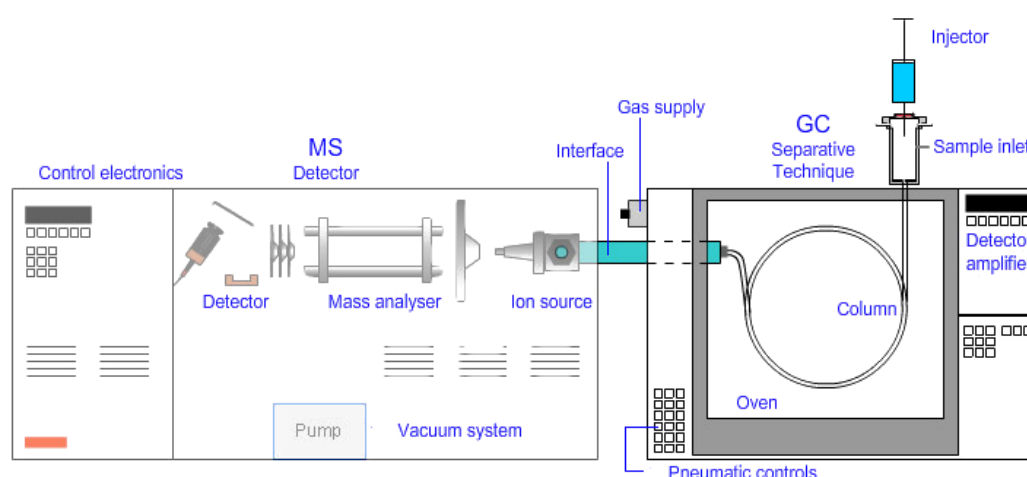
In this project, sample preparation and extraction methods were developed and optimised as explained in the previous chapter. Direct LLE of the hair digest selectively and efficiently isolated THC, CBD, CBN and 11-OH-THC, and the extract was derivatised using BSTFA with 1% TMCS and analysed using GC-EI-MS. Extraction of the hair digest using SPE after pH adjustment produced clean extracts with reasonable recovery for THC-COOH, when derivatised with HFIP-TFAA and analysed using 2D GC-NCI-MS. Before these two analytical methods can be employed for the quantitative determination of cannabinoids in hair samples from cannabis users, they have to be validated to ensure they are fit for purpose. Many organisations or scientific societies have published guidelines or recommendations for method validation aiming at ensuring high quality and reliable data. These have included The Food and Drug Administration (FDA) Guidance for industry-bioanalytical method validation (2001) (170), Society of Forensic Toxicologists/the American Academy of Forensic Sciences (SOFT/AAFS) forensic toxicology laboratory guidelines (2006) (5), the United Kingdom and Ireland Association of Forensic Toxicologists (UKIAFT) forensic toxicology laboratory guidelines (2010) (171), and the standard practices for method validation in forensic toxicology which was published by the Scientific Working Group for Forensic Toxicology (SWGTOX) in May 2013 (172). According to the latest SWGTOX guidelines, validation parameters vary depending on whether the method will be used for qualitative or quantitative analysis. In the case of a qualitative method, only a few parameters are subject to investigation including; the limit of detection, interference study, carryover and stability of analyte in given matrices. For quantitative methods, where concentrations are to be reported, additional parameters are subject to investigation including; linearity, bias and precision, limit of quantification.

## 4.2 Aims

The aim of work presented in this chapter is to describe the two instruments acquisition parameters, principles of keys components in the 2D GC-NCI-MS, included establishing the approximate retention time on the primary column, the Dean's switch cut time, the GC oven and cryo-focusing trap settings, the retention times on the secondary column and the correct ions to monitor using SIM. Moreover, to quantitatively validate the GC-EI-MS method for quantitation of THC, CBD, CBN and 11-OH-THC and 2D GC-NCI-MS for quantitation of THC-COOH using fortified hair matrices. Sample preparation was carried out using the optimised procedure reported in the previous chapter.

## 4.3 Gas chromatography–mass spectrometry (GC-MS)

Gas chromatography-mass spectrometry (GC-MS) is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample. GC-MS has several applications including but not limited to drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples. It has been widely regarded as a "gold standard" for forensic substance identification due to its high specificity. The main component of GC-MS is shown in Figure 4-1.



**Figure 4-1 Schematic diagram showing the main components of standard GC-MS system**

The main two components of the GC system are the inlet (or injector) and the capillary column. The main role of inlet is to transfer the sample to the capillary column by converting the analytes into its volatile state and introducing it into a continuous flow of carrier gas, typically, helium. The most popular inlet for capillary column gas chromatography is the combined split/splitless inlet. The other GC component is the capillary column. The main function of the GC column is to separate the different components of a mixture based on their polarities. This causes each compound to elute at a different time, known as the retention time (RT) of the compound. Capillary column is composed of tubing, usually made of fused silica and stainless steel, and stationary phase. There are many stationary phases. Most are high molecular weight, thermally stable polymers that are liquids or gums. The most common stationary phases of this type are the polysiloxanes and polyethylene glycols. The most basic polysiloxane is the 100% methyl substituted. When other groups are present, the amount is indicated as the percent of the total number of groups. For example, a 5% diphenyl-95% dimethyl polysiloxane contains 5% phenyl groups and 95% methyl groups. For GC-MS, a "MS" version is available commercially and is claimed to provide low column bleeding into the MS. This is achievable by strengthening polymer siloxane backbone by incorporating phenyl or phenyl-type groups. An ultra-inert version is also recently available and is claimed to provide the lowest column bleed and highest column inertness for a wide range of analytes, including active compounds and trace level samples. DB-5MS, HP-5MS UI, DB-17MS are the columns employed in the work presented in this chapter and are all examples of these columns. The two dimensional GC-MS (2D GC-MS or GCxGC-MS) is an advanced version of the conventional GC-MS. This instrument employs a pair of GC columns, usually of different polarities, connected in series through a modulator. All effluent from the first column (comprehensive) or part of it (heart-cut) is trapped in the modulator for a fixed period of time before being focused and injected into the second column.

The effluent from the standard GC-MS or 2D GC-MS, carrying the analytes of interest, passes through the transfer line (interface) and enters into the mass spectrometer. The three main components of the MS are the ionisation source, analyser and detector. The most common form of ionization sources is electron ionization (EI), also called electron impact. In this ionisation method, molecules

that arrive into the source body are bombarded with free electrons, emitted from a filament, causing the molecule to fragment in a characteristic and reproducible way. This "hard ionization" technique produces fragments of low mass to charge ratio ( $m/z$ ) and few, if any, molecular ions reach the molecular mass unit. Typically, 70 eV (electron Volts) is the electron energy applied to the system in the EI source. The common usage of 70 eV as the electron energy enables spectra comparison with spectra libraries software usually integrated into the analysis system and provided by the manufacturer. Examples of most known sources of libraries include National Institute of Standards (NIST), Wiley, the American Academy of Forensic Scientists (AAFS).

Chemical ionisation (CI) is a softer ionisation method than electron impact (EI). The layout of a chemical ionization ion source is shown in Figure 4-2. The principle of CI is transferring electron, proton, or other charged species from the reagent gas to the neutral molecule of interest in its gaseous phase. In negative chemical ionization (NCI) mass spectra are obtained by deprotonation of acidic analytes like THC-COOH. Electron capture (EC) or electron attachment is one process of special interest when performing NCI. Its importance comes from the superior sensitivity it provides with many analytes. In EC, a freely moving external electron, not provided by reagent gas, is incorporated into the orbital of the molecule and EC occurs when these electrons approach their thermal energy.

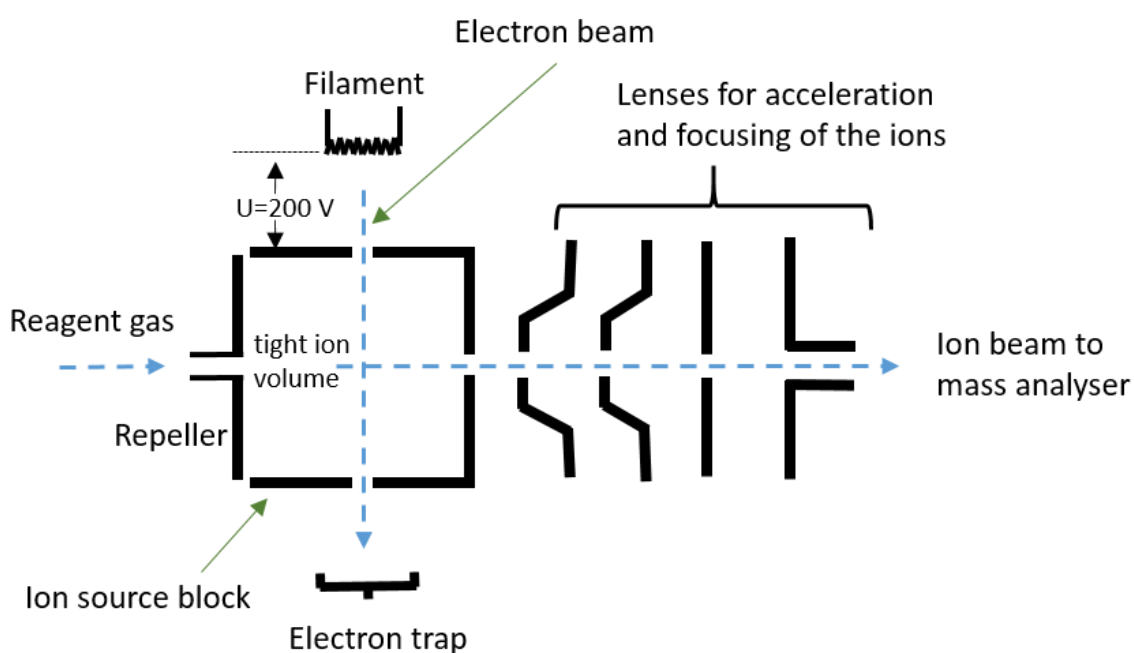


Figure 4-2 Schematic layout of a chemical ionization ion source.

Mass analyser is component of MS responsible for filtering sample ions, based on their mass-to-charge ratio ( $m/z$ ). The most common mass analysers employed in the MS is the single quadrupole (SQ). SQ consists of four cylindrical rods, set parallel to each other and connected together electrically. A radio frequency (RF) voltage with a DC offset voltage is applied between one pair of rods and the other. Ions produced by ionisation source will travel down the quadrupole between the rods, however, only ions of certain  $m/z$  will reach the detector for a given ratio of voltages and other ions will collide with the rods. The mass analyser is often operated in two modes; full-scan (FS) and selected ion monitoring (SIM). When collecting data in FS mode, a target range of mass fragments, usually dictated by molecular weight of targeted analytes or derivatives, is determined and put into the instrument's method. Typically, a range of 50-400  $m/z$  would be monitored. The sensitivity of the instrument in FS mode is decreased due to performing fewer scans per second since each scan will have to detect a wide range of mass fragments. The two main uses of FS mode are the identification of unknown substances in a sample and to help in instrument method development during early stages by determining the retention time and the mass fragment fingerprint of a particular analyte before moving to a SIM mode.

In SIM mode, much lower detection limit is achievable as only certain ion fragments of good intensities, selected from FS spectrum, are targeted. This means that the analyser is only looking at a small number of ions during each scan. The final component of the MS is the detector. The detector role is to record either the charge induced or the current produced when an ion passes by or hits a surface. Typically, some type of electron multiplier is often used.

## **4.4 GC-EI-MS**

### **4.4.1 Analytical instrument parameters**

The GC-MS system used for analysis of THC, CBD, CBN and 11-OH-THC is an Agilent GC model 6890 Series coupled with an Agilent 5975N mass selective detector. Cannabinoid TMS derivatives were separated on a fused silica capillary column DB5-MS+DG (30 m length x 0.25 mm (ID) x 0.25  $\mu\text{m}$  film thickness). The oven temperature program is shown in Table 4-1. The temperatures for the injection

port, ion source, quadrupole and interface were set at 250, 230, 150 and 280 °C, respectively. Split injection was used with a split ratio of 1:1 to inject 1 µl. The flow of the carrier gas (helium) through the column was set constant at 1.0 mL/min. To determine the retention times and characteristic mass fragments, electron impact (EI) mass spectra of the analytes were recorded by total ion monitoring. For quantitative analysis the chosen diagnostic mass fragments were monitored in selected ion monitoring (SIM) mode  $m/z$ : 371, 386, 303 for THC-TMS; 390, 337, 458 for CBD-di-TMS; 367, 368, 382 for CBN-TMS, 371, 474, 459 for 11-OH-THC di-TMS; and 374, 389 for THC-TMS- $d_3$  as internal standard for THC, CBD and CBN, and 374, 462 for 11-OH-THC 2-TMS- $d_3$  as internal standard for 11-OH-THC. For quantification, peak area ratios of quantifier ions of the analytes to the internal standard (underlined) were calculated as a function of the concentration of the substances. The MSD was used in the electron impact mode at 70 eV. The MSD was auto-tuned weekly with perfluorotributylamine. Data acquisition and analysis were performed using standard software supplied by the manufacturer (Agilent ChemStation).

**Table 4-1 Oven temperature program of GC-EI-MS**

Initial temperature: 80 °C		Initial time: 1.00 min
Ramps:		
Rate (°C/min)	Final temp	Final time
30	270	5.00
10	300	5.00
Run time: 20.33		

#### 4.4.2 Initial testing of the method

In order to identify the retention times and mass fragments of each compound and its deuterated ISTD, 100 µl of CBD, CBN, THC, 11-OH-THC working standard solutions and 100 µl of THC- $d_3$  and 11-OH-THC- $d_3$  working standard solutions all at 1 µg/mL were transferred into a vial. The solvent was evaporated to dryness under a stream of nitrogen at room temperature and the residue was derivatised using 50 µl BSTFA with 1% TMCS at 80 °C for 20 minutes. This was then transferred to the GC auto sampler vial and injected for analysis using the GC-MS method which was routinely used in the Forensic Medicine and Science laboratory,



University of Glasgow for the identification and measurement of THC and THC-COOH in blood. Analyses were performed using FS mode to identify the retention times and mass fragments of each compound and their deuterated ISTD. Examples of mass spectra and chromatograms are shown in the previous chapter. Prior to carrying out a full validation study, the method was tested with full calibration including several points covering the expected LOD and LOQ ranges and quality control standards. The silanised high recovery vials were used as the derivatisation and injection medium. There were two major issues from this initial testing that were subsequently evaluated and overcome.

- Co-eluting background interferences
- Detector saturation at high calibration curve points

#### **4.4.2.1 Co-eluting background interferences**

There was co-eluting background interference present on the CBD peak at  $m/z$  337. These interferences affected the ratios of the quantitation and qualifier ions at the lower concentrations. The interferences were observed on the calibration standards. The source of the background interferences was investigated and it was determined that the background interferences originated from the hair matrix. It was not possible to eliminate the interference. The ion at  $m/z$  301 was assessed as a possible alternative qualifier ion, however, the same interference problem was encountered. Therefore, to rectify this, the ion at  $m/z$  337 was kept as the qualifier ion as the interference was affecting only lower concentrations. The ion at  $m/z$  458 was used for monitoring the ion ratio instead with the quantifier ion at  $m/z$  390. In addition, the LOQ was later elevated to 10 ng instead of 8 ng to ensure a consistent ratio at the lower concentrations.

#### **4.4.2.2 Detector saturation at high calibration curve points**

The limit of detection (LOD) was within the expected range (0.04 - 0.2 ng/mg) for all analytes. However, the calibration curves for all analytes were found not to give the good linearity that was established earlier using the 7 mL vial as a derivatisation medium. Due to the wide range of concentrations reported in the literature for cannabinoids in hair, and also the lack of data on the concentrations present in hair of targeted ethnicity, a wide calibration range was proposed.

Primarily, up to 400 ng/50mg of hair (equivalent to 8 ng/mg) was proposed. However, detector saturation encountered with analytes at high concentrations was found to affect the linearity and did not allow such a wide calibration. Detector saturation for all analytes started at 150 ng/50mg. Therefore, as a compromise, and to avoid other solutions may involve changes in the optimised sample preparation method, such as increasing the volume of the derivatisation reagent to reduce the signal, the impact of applying different split ratios on both low and high ends of the calibration were tested using a trial and error approach. It was found that a split ratio of 1:1 maintained the LOD and LOQ within the expected range and slightly improved the linearity calibration range up to 200 ng/50 mg for CBN and 11-OH-THC and 300 ng/50 mg for THC and CBD.

## **4.5 2D GC-NCI-MS**

### **4.5.1 Historical overview on the MDGC**

According to the classical terminology in column chromatography, separations are commonly called two- or multidimensional (2DGC or MDGC) when separations of all or certain selected groups of sample components are repeated in two or more columns of different polarity, which are coupled in series to the column in which the first separation was carried out. The separation is called ‘heart-cutting’ when only one or more chosen elution regions is sent to the second column for further separation, and ‘comprehensive’ when the entire chromatogram eluting from the primary column is submitted to the secondary column. The first demonstration of MDGC was reported by Simmons and Snyder in 1958 (173). This early version involved ‘Heart-cutting’ analysis by connecting two or more gas-liquid chromatographic columns in such a manner that preliminary cuts prepared by the first column could be charged directly to one or more secondary columns. Ten years later, Dean invented a switching device that enables transferring a portion of the GC run onto a secondary column using the gas flow (174). A significant development in the technique arose in the early 1990s, when comprehensive two-dimensional gas chromatography was proposed by Phillips and Liu (175). Since then, it has undergone a reasonably predictable development.

Many review articles have been published reviewing different aspects relating to MDGC. In 1994, Schomburg reviewed the principles, instrumentation and methods

for some typical applications of MDGC (176). Phillips and Beens summarise the development of comprehensive MDGC instrumentation and applications up to 1999 (177). A year later, in a two-part review article, Wolfgang reviewed concepts, instrumentation, and applications of the conventional ‘heart-cutting’ MDGC (178) and the comprehensive MDGC (179). In 2002, Pursch *et al* published a review on modulation techniques and applications in comprehensive two-dimensional gas chromatography. Kueh *et al* reviewed, in 2003, the application for 2D GC to the analysis of drugs in doping control (180). In another two-part review, Adahchour *et al* reviewed, in 2006, recent developments in comprehensive MDGC including instrumental set-up in part 1 (181) and modulation and detection in part 2 (182). The same group published, two years later, a review summarising the recent developments in the applications of comprehensive MDGC (183). The Marriott *et al* review, published in 2012, provided a technical overview of recent method implementation for both the heart-cut MDGC and the comprehensive MDGC (184). The two most recent reviews in the literature for applications and future prospects of MDGC techniques were by Tranchida *et al*, in 2012, for Heart-cutting MDGC (174), and Edwards *et al*, in 2015, for the comprehensive MDGC (185).

It was noticed that the symbols and abbreviations for the two types of MDGC are inconsistent amongst researchers. For instance, Wong *et al* have described the abbreviation (GC-GC) to refer to the MDGC in ‘heart-cutting’ mode and (GCxGC) to refer to the comprehensive version (186). However, in an earlier publication, Phillips *et al* used the abbreviation (GC-GC) to refer to the comprehensive version. Other publications such as that by Moore *et al* and Guthery *et al* have used the abbreviation (GCxGC) to refer to the two-dimensional GC separation of any type (141,157). In 2003 Schoenmakers *et al* published a position paper defining various nomenclature and conventions in comprehensive MDGC (187). Therefore, the abbreviation (GC-GC) will be used throughout to describe the techniques employed in this work ‘heart-cutting two-dimensional GC’.

Applications of MDGC have ranged from petroleum to environmental and biological samples analysis. Despite the fact that MDGC approaches are available for a long period, they are still not widely employed in the field of bioanalysis. This, possibly, reflects the general preference of standard GC over the MDGC. MDGC techniques have been reported in only in few publications for analysis of

cannabinoids in different biological matrices. Moore *et al* developed a method to detect THC-COOH in hair by using GC-GC in combination with electron capture chemical ionization (ECCI) MS. To enhance the ECCI detection, THC-COOH was derivatised using fluorinated acylation agents. The measured detection concentration was 0.05 pg/mg hair (141). In the subsequent year, Marin's group reported a new method to detect the two metabolites THC-COOH and 11-OH-THC in meconium using GC-GC-MS (188). The method was validated and trace amounts of THC-COOH were detected in ten samples from babies born to drug-abuse patients. The separation in the secondary column was necessary as the targeted analytes co-eluted with other interfering substances during the first-column separation. Milman *et al* reported a combination of SPE and GC-GC-MS for the quantitative determination of THC, CBD, CBN, 11-OH-THC and THC-COOH in oral fluid (189). They used NCI for the detection of THC-COOH after derivatization with TFAA and HFIP; the other analytes were detected using EI after derivatization with BSTFA. Jones *et al* reported a method for quantitation of THC-COOH in 60 matched pairs of hair and fingernail (158). Samples were first digested using NaOH and were subject to SPE extraction and derivatisation with HFIP-PFPA. Analysis was achieved using GC-GC equipped with a low thermal mass (LTM) Series II System and tandem MS operated in the NCI mode.

#### 4.5.2 Analytical instrument parameters

The GC-MS system used for analysis of THC-COOH was an Agilent GC model 6890 Series coupled with an Agilent 5975N mass selective detector. THC-COOH derivatives were separated first on a low polarity column ultra-inert HP5-MS (30 m length x 0.25 mm (ID) x 0.25 µm film thickness). A cut containing the analyte of interest was determined and transferred into the secondary analytical column. The secondary column was of medium polarity DB-17MS (15 m length x 0.320 mm (ID) x 0.25 µm film thickness). The oven temperature program is shown in Table 4-2. The temperatures for the injection port, ion source, quadrupole and interface were set at 250, 106, 150 and 280 °C, respectively. The injection volume was 1 µl of sample. Pulsed splitless injection was used with 50 psi pulse pressure and 0.80 min pulse time. The pressure of the carrier gas (helium) through the primary and secondary columns was set constant at 30.39 and 7.26 psi, respectively. At these pressure values, the flow in the primary and secondary columns is calculated to be about 2 mL/min in the primary column, and 3 mL/min in the secondary column.

The primary detector was flame ionisation detector (FID) and was set at 250°C with the hydrogen and air flows at 40 mL/min and 450 mL/min, respectively.

The secondary detector was MS operated in NCI mode with a gain factor of 14. To determine the retention times and characteristic mass fragments, mass spectra of THC-COOH and its deuterated ISTD derivatives were recorded by total ion monitoring. For quantitative analysis the chosen diagnostic mass fragments were monitored in the selected ion monitoring (SIM) mode  $m/z$ : 422, 590 for THC-COOH derivative; and 425, 593 for THC-COOH- $d_3$  as IS. For quantification, peak area ratios of quantifier ions of the analytes (underlined) to the internal standard were calculated as a function of the concentration of the substances.

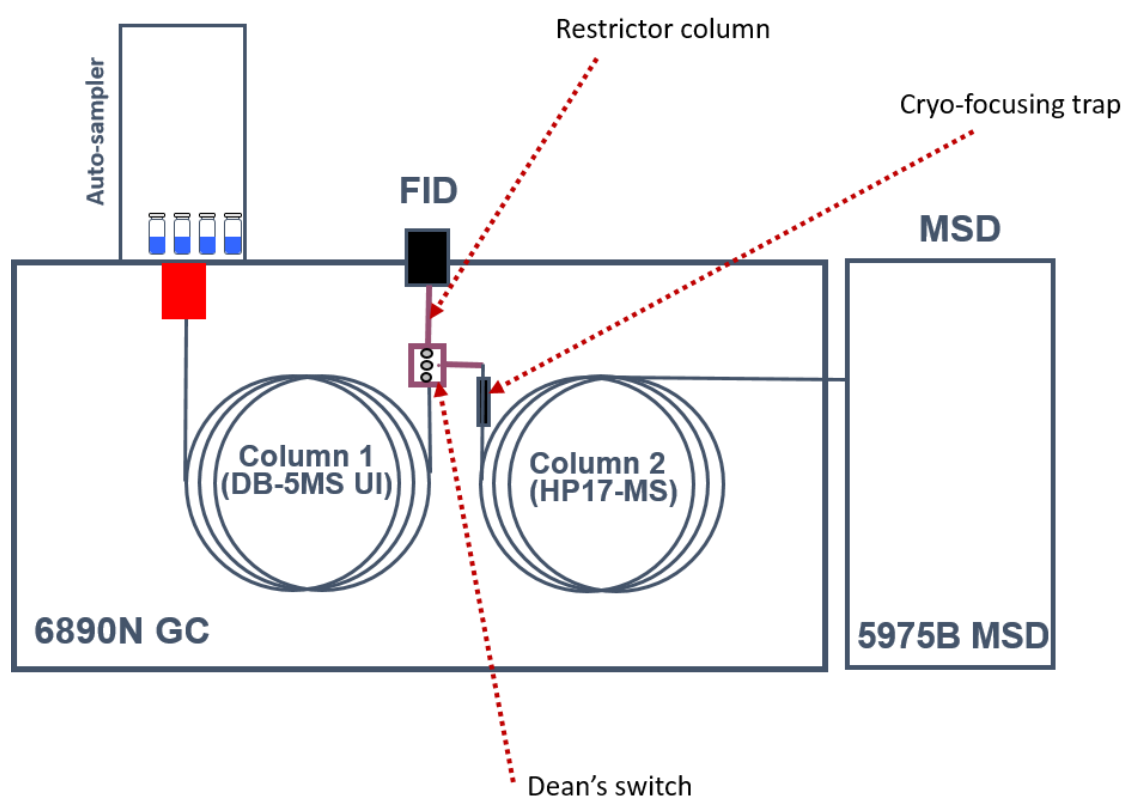
For the ionisation source, different gases can be used as the reagent or collision gas, such as, methane and ammonia. In this method ammonia, with 35 mL/min flow rate, was chosen due to its superior thermalizing power over methane. Despite the fact that use of ammonia has some advantages, it has also some drawbacks. The use of ammonia affects the maintenance requirements of the mass spectrometer detector (MSD). Typically resulting in the need to clean the source more often, as well as requiring more frequent replacement of the rough pump oil. When the pressure of the ammonia supply exceeds 5 psi, it will start condensing from a gas to a liquid. It tends to break down vacuum pump fluids and seals also and to prevent and minimize the pump damage, shutting off the CI gas after each run and more frequent vacuum system maintenance becomes necessary.

The MSD was auto-tuned weekly. Data acquisition and analysis were performed using standard software supplied by the manufacturer (Agilent ChemStation).

In addition to the secondary analytical column, conversion of an Agilent 6890 gas chromatograph into a heart-cutting GC system required the addition of another two components: the modulator and cryo-focusing trap. Hardware configuration of GC-GC-MS system is shown in Figure 4-3. The operating parameters for the Dean's switch, determination of cut time and cryo-focusing trap will be covered in the sections 4.5.3, 4.5.4 and 4.5.5

**Table 4-2 Oven temperature program of GC-GC-NCI-MS**

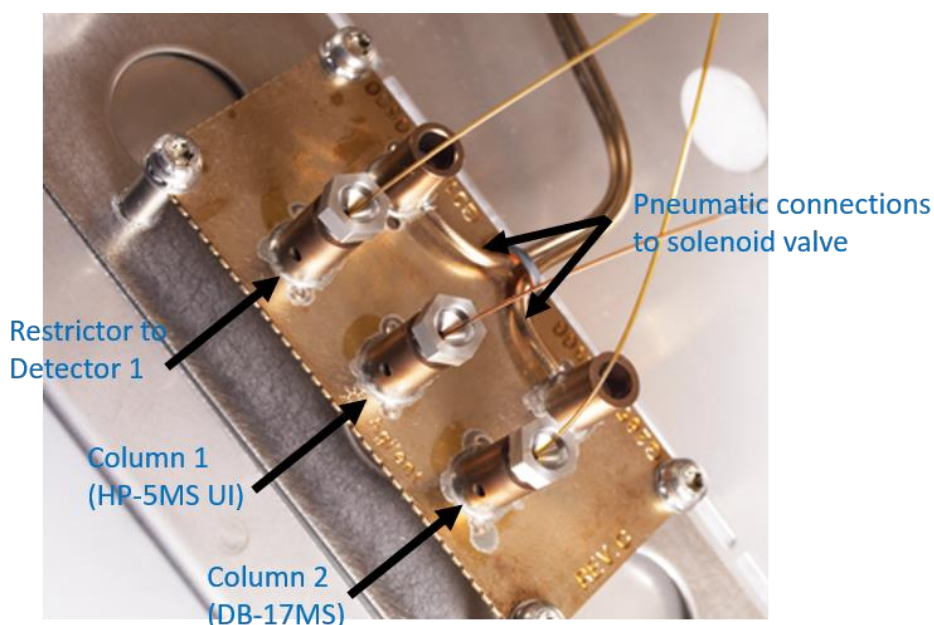
Initial temperature: 150 °C      Initial time: 1.00 min		
Ramps:		
Rate (°C/min)	Final temp	Final time
50	220	0.00
10	263	0.00
120	180	0.00
10	230	0.00
Post temp: 320 °C		
Post time: 2.00 min		
Run time: 12.39		



**Figure 4-3 Schematic representation of an Agilent two dimensional 6890N gas chromatograph (GC) coupled with a 5975B mass selective detector (MSD), flame ionization detector (FID) and an auto-sampler.**

### 4.5.3 Dean's switch

As mentioned earlier, Dean's Switch technology has been in use since 1967. However, the classical configurations that employ traditional rotary valves, stainless-steel tubing and fittings have a high thermal mass and do not track oven temperature very well, and hence are susceptible to leaks over time and can cause peak broadening. The employed Dean's switch in this work is a recently innovated chromatographic device by Agilent, called Capillary Flow Technology (CFT), and is claimed to eliminate these chromatographic problems. Agilent's Dean's Switch combines individual switch components into a single, smaller device with a three-port manifold connected pneumatically to a solenoid valve as shown in Figure 4-4.



**Figure 4-4 Photo of Capillary Flow Technology Dean's Switch.**

In addition to the two columns, a deactivated restrictor tubing (0.18 mm ID) was connected to the Dean's switch at one end and was attached to the primary detector FID at the other end. After determination of the desired method parameters (column dimensions, temperature, flows, etc.), the Dean's switch calculator software is often employed to calculate the restrictor length and pressure set points to operate the system. A screenshot of the Agilent Dean's switch calculator is shown in Appendix III. In the method developed for this work, the restrictor length was 0.751 m. The Pressure Control Module (PCM) is the preferred switching gas source, as it automatically compensates for changes in atmospheric pressure, however, it was not possible to build in a PCM as the GC

second inlet was already occupied. The three channel Aux Pressure controller was used instead and was programmed to operate at a pressure of approximately 10 psi. This supplied switching gas allowing all the flow from the primary column to vent through the restrictor column to the FID until the time of THC-COOH elution. The Dean's switch was then turned on to allow the carrier gas to enter the secondary analytical column, which has different separation properties, for 0.18 minutes. The switch times have to be very short due to the low internal volume of the pneumatic coupler (190). The flow was then redirected to the FID vent until the end of the analysis. Determination of the cut time is explained further in section 4-5-4. Conventionally, this method is used to separate compounds that do not separate on the primary column, however, with dirty matrices such as hair, it is a practical option to get rid of potential matrix effect and interferences. The basic layout of the Dean's switch and flow directions is shown in Figure 4-5.

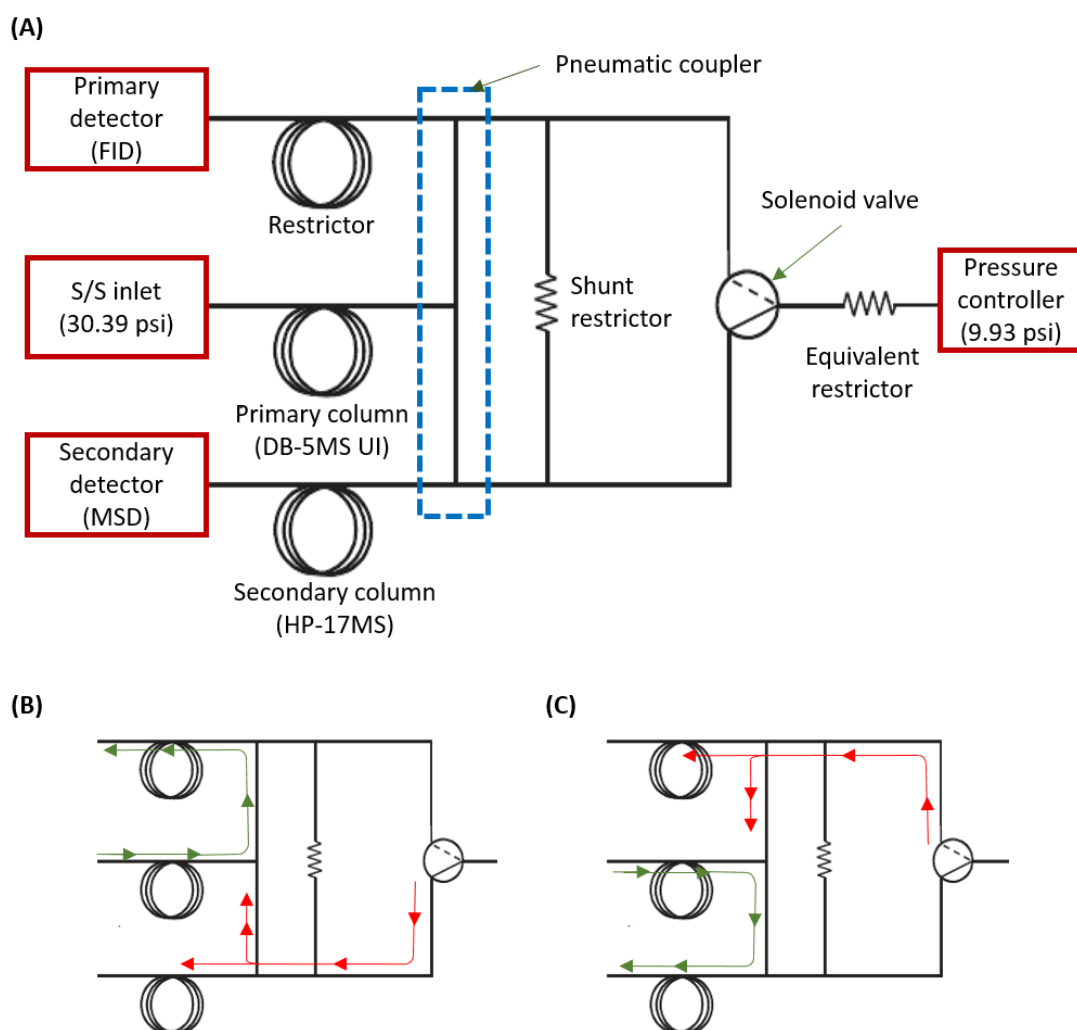
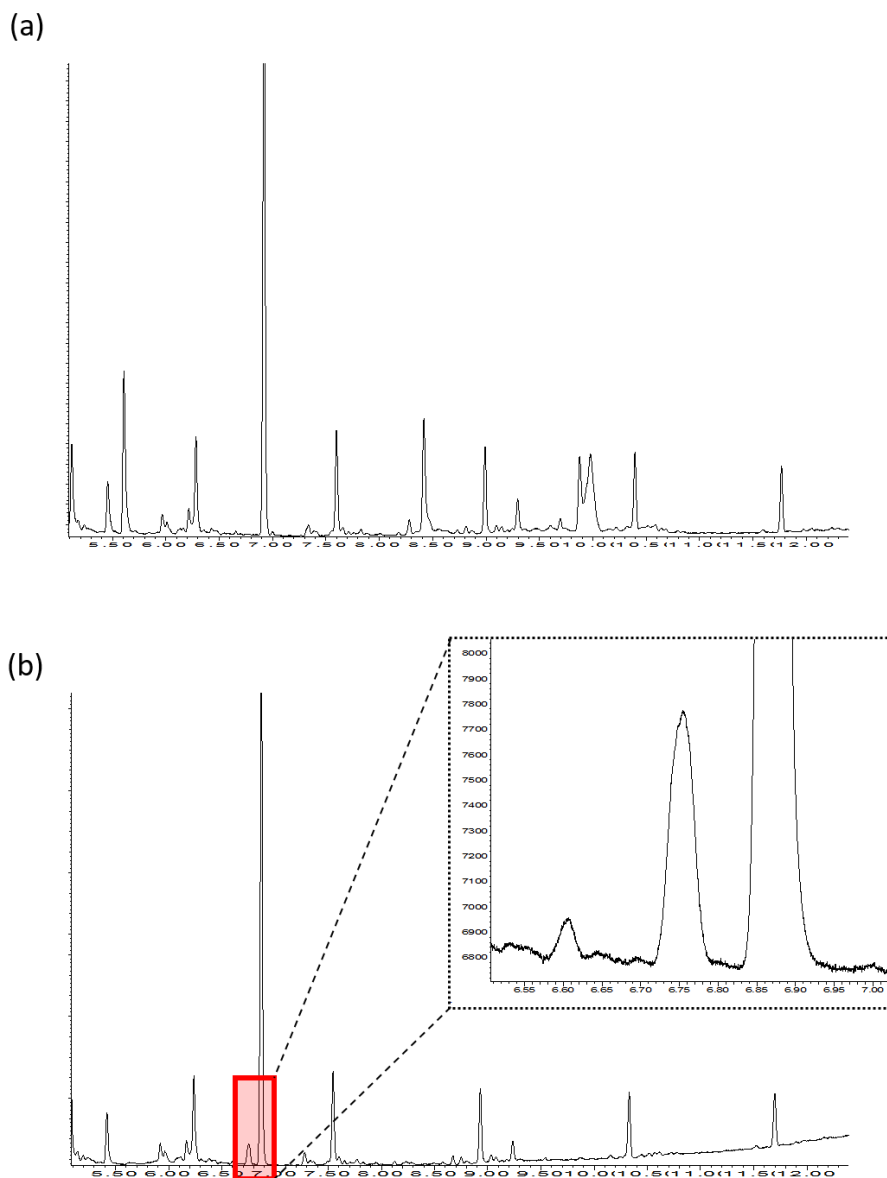


Figure 4-5 (A) Dean's switch basic layout, and the flow direction when the valve is switched OFF (B) and ON (C). Adopted from ref (190).



#### 4.5.4 Determination of Dean's switch cut times

It was important to make sure that the Dean's switch transferred the whole analyte to the secondary column. A partial cut of the first column eluent will hugely affect the sensitivity. Therefore, a blank methanol and a high concentration mixture of THC-COOH and its deuterated ISTD (100  $\mu$ L of 1  $\mu$ g/mL working solution for both analyte and IS) were dried under a stream of nitrogen and derivatised with 50  $\mu$ L TFAA and 30  $\mu$ L HFIP. The mixture was incubated at 80 °C for 20 minutes, and then injected. The signals were monitored, primarily, on FID and then on MS detector. The two FID chromatograms of the blank and drug were compared against each other as shown in Figure 4-6.



**Figure 4-6** FID chromatograms of unextracted, derivatised blank (a) and 100 ng of THC-COOH and its internal standard (b) Enlarged area shows the THC-COOH derivative peak retention time 6.75 min.

A peak with retention time (RT) 6.75 minutes was observed only in the chromatogram that contained the analyte of interest. The peak width was 0.11 minutes (starts at 6.71 and finishes at 6.80 minutes). Based on this result, the Dean's switch valve was programmed to cut from 6.65 to 6.83 (0.18 minutes) using ChemStation software. This very narrow cut was chosen to avoid transferring the, untargeted peaks, which were eluting close to analyte of interest, to the secondary column. Moreover, to eliminate the possibility of overloading the pneumatic coupler. This cut is flexible to minor changes in retention time, however, it must be re-evaluated after carrying out a major maintenance such as cutting the column which may result in a significant decrease of RT. This accuracy of cut was confirmed further by carrying out MS analysis.

#### 4.5.5 Cryo-focusing trap

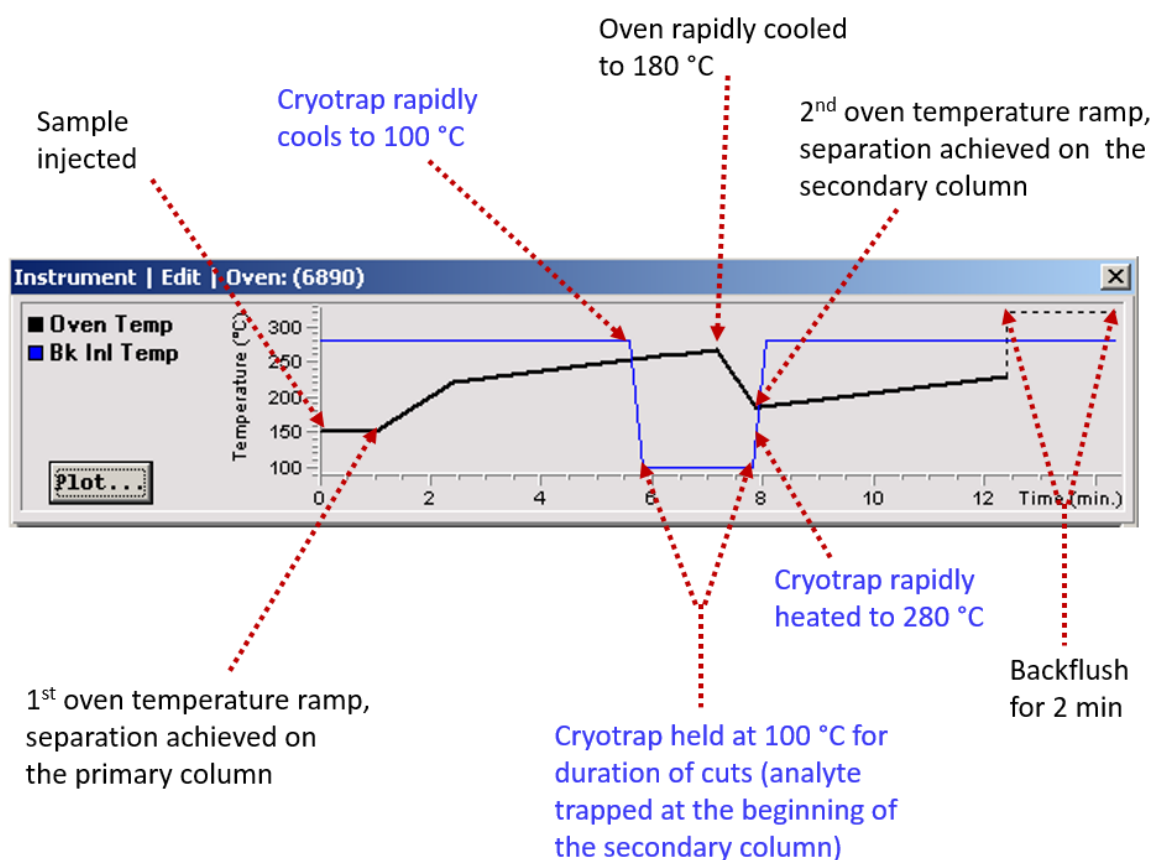
The Cryo trap is installed at the beginning of the secondary column. It is designed to efficiently trap eluents from a capillary GC separation with the use of appropriate cryogens to cool the trap, and then to re-vaporise the trapped components for subsequent capillary GC separation using rapid but controlled heating of the trapping zone. The trap settings in this work were established based on the cut time that was determined previously as explained in section 4.5.4. The settings were controlled and dealt with in ChemStation as back inlet and are shown in Table 4-3.

Table 4-3 Cryo trap settings

Initial temperature: 280 C    Initial time: 5.60 min		
Ramps:		
Rate (°C/min)	Final temp	Final time
800	100	2.00
800	280	0
Cryo type: Nitrogen		
Pressure: 7.26 psi		
Gas type: Air		

The aim of using a cryo trap with the two dimensional GC analysis is to focus the analytes at the start of secondary column which is believed to improve the sensitivity and peak shape. To cool down the column from 280 to 100 °C at a rate

of 800 °C/minute, we need only 0.225 minute (time = distance / speed => time = 180 / 800 = 0.225 minutes). However, starting the trap one minute earlier ensures that the trap is cold before the THC-COOH derivative arrival. To attain the best focusing result, the second oven must be kept at a low temperature during heart-cutting. This explains the fast ramp-down described previously in Table 4-2 at the maximum rate 120 C/min. Figure 4-7 is a screenshot from Agilent ChemStation Software illustrating combined GC oven and cryo-focusing trap settings for the duration of the analysis time.



**Figure 4-7 Screenshot from Agilent ChemStation Software illustrating the GC oven (Oven temp) and cryotrap (Bk Inl Temp) settings for duration of the analysis time**

#### 4.5.6 Backflush

One of the advantages of Dean's switch is that it enables basic MDGC operations such as backflushing. Backflush can be defined as reversal of flow through a column which leads to forcing late-eluting sample components back out the inlet end of the column. Therefore, backflush capabilities can significantly reduce GC run times and prolong column life. In this work, post-run backflush was programmed within the analytical run; post-run temperature was increased to 320

°C and held for 2 min, while column 1 and 2 pressures were ramped rapidly to 1 and 50 psi, respectively.

#### **4.5.7 Initial testing of the method**

Initially, retention times and mass fragments of THC-COOH and its deuterated ISTD, THC-COOH-d<sub>3</sub> were confirmed as described previously in chapter 3. Prior to carrying out a full validation study, the method was tested with full calibration including several calibrators covering the expected LOD and LOQ ranges as well as quality control standards. Calibrator and QC standards were prepared as shown in Table 4-4. The silanised high recovery vials were used as the derivatisation and injection medium. Initially, detection sensitivity was found not to provide sufficient identification criteria within the expected range (0.1 -2 pg/mg) with extracted hair samples. Different attempts were carried out to improve the detection sensitivity, it was possible to improve the detection sensitivity significantly employing an ultra-inert column as the primary separation column. The instrument was able to detect as low as 10 pg total with a S/N ratio > 10 when unextracted standards were injected.

### **4.6 Materials and Methods**

#### **4.6.1 Materials**

These were previously described in chapter 3, section 3.3..

#### **4.6.2 Preparation of calibrations standards**

Working solution mixture (WS#1) at 1 µg/mL for THC, CBD, CBN, 11-OH-THC and THC-COOH was prepared as described in section 3.3.3. An aliquot of this solution was used to prepare a working solution (WS#2) at 10 ng/mL by 1:100 dilution. An aliquot of WS#2 was used to prepare a third working solution (WS#3) at 1 ng/ml by 1:100 dilution. Stock and working solutions were stored at -20 °C. Calibrators were freshly prepared for each analysis from these working calibrant solutions by adding the appropriate volume to approximately 50 mg of drug- free hair in a glass extraction tube. The calibration procedure is shown in Table 4-4.

#### 4.6.3 Preparation of internal standard solution

A working solution of 1 µg/ml was prepared for THC-*d*3, 11-OH-THC-*d*3 and THC-COOH-*d*3 as described in section 3.3.4. Internal standard concentration was chosen to be equivalent to the medium QC sample.

#### 4.6.4 Preparation of quality control standards

Quality control samples (QCs) were prepared and analysed in addition to calibration standards and specimens to carry out bias and accuracy calculations. QCs were also tested later with case samples batches to ensure that accurate results were being obtained for the specimens. The working solutions used for preparation of QCs were different from the calibration standards but were prepared the same way. Stock and working solutions were stored at -20 °C. Three QC levels were freshly prepared for each analysis by adding an appropriate volume of the working control solutions to approximately 50 mg of drug-free hair in a glass extraction tube. The procedure of preparing QC samples is shown in Table 4-4.

**Table 4-4 The preparation of limit of detection (LOD), limit of quantitation (LOQ), calibration standards containing  $\Delta^9$ -tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN), 11-hydroxy- $\Delta^9$ -tetrahydrocannabinol (11-OH-THC), 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) and low, medium and high quality control samples for the validation studies.**

Validation studies:	Analytes	Working solutions	Volume (µl)	pg total		equivalent pg/mg		Comments			
THC, CBD, CBN, 11-OH-THC, THC-COOH	1 ng/mL (WS#3)	5	5	2D GC-NCI-MS (THC-COOH)	LOD & LOQ	0.1					
		10	10			0.2		SoHT recommended LOQ			
		15	15			0.3					
		20	20			0.4					
		25	25			0.5		Expected LOD			
		50	50			1		Expected LOQ			
		75	75			1.5					
		100	100			2					
		150	150		3	Low QC					
		200	200		4						
	10 ng/mL (WS#2)	30	300		CALIBRATION	6	IS level	Medium QC			
		50	500			10					
		80	800			16		High QC			
		100	1000			20					
			ng total				equivalent ng/mg				
		100	1			0.02					
	1 µg/ml (WS#1)	200	2	GC-EI-MS (THC, CBD, CBN, 11-OH-THC)	LOD & LOQ	0.04		Expected LOD for THC			
		4	4			0.08		Expected LOQ for THC			
		6	6			0.12		SoHT recommended LOQ for THC			
		8	8			0.16					
		10	10			0.2		Low QC			
		20	20			0.4					
			50		50			CALIBRATION	1		
			100		100				2	IS level	Medium QC
			200		200				4		
			300		300				6		High QC
			400 µl		400				8		

## 4.7 Method validation experiments

### 4.7.1 Interference Studies

The interferences can be defined as the non- targeted compounds. According to Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation, interference studies must be carried out to evaluate the ability of the analytical method to distinguish the target analyte in a complex matrix from interferences from common sources (172). Three main sources of interferences were evaluated. Firstly, matrix interferences were evaluated by analysing 10 hair samples from different donors using the final extraction method and instrumentation to demonstrate the absence of common interferences from the matrix by comparing the chromatograms. Secondly, another possible source of interferences is the stable-isotope internal standards. The deuterium-labelled internal standards may contain the non-labelled compound, as an impurity, which could impact on quantitation results of its non-labelled analogue. Therefore, 100 µl of each unextracted internal standard at 1µg/ml was derivatised with BSTFA or TFAA-HFIP and injected into the corresponding analytical system. The signals of the analytes of interest were then monitored. Thirdly, the most important source of interferences is the endogenous drugs or metabolites that may be present in the case sample due to exposure or active consumption. Therefore, a wide range of commonly encountered analytes were evaluated by injecting neat reference materials after derivatisation into the system. Appendix IV shows a list of 91 compounds included in the study.

### 4.7.2 Limits of Detection (LOD) and Limits of Quantification (LOQ)

LOD can be defined as the estimation of the lowest concentration of an analyte in a sample that can be reliably differentiated from blank matrix and identified by the analytical method. LOQ is an estimate of the lowest concentration of an analyte in a sample that can be reliably measured with acceptable bias and precision. There are a number of different approaches for determining the LOD and LOQ proposed in SWGTOX guidelines. The selected approach in this study was to estimate the LOD and LOQ using background noise. 40-50 mg of pooled blank hair from at least three sources was spiked with decreasing concentrations. These were extracted and analysed in duplicate (two separate samples) over a minimum

of three runs. Instrument LOD is considered the lowest concentration that gives a reproducible instrument response and which also can be distinguished from the matrix background noise with signal to noise ratio ( $S/N$ )  $\geq 3$ , while LOQ is considered the lowest concentration that gives a reproducible instrument response with  $S/N$  ratio  $\geq 10$ .  $S/N$  ratios were calculated using ChemStation software. These can be calculated manually using the following Equation 4-1:

Equation 4-1

$$S/N = \frac{\text{height of analyte}}{\text{amplitude of noise}}$$

The LOD and LOQ of the GC-EI-MS method for THC, CBD, CBN and 11-OH-THC were determined by spiking 50 samples of pooled blank hair with decreasing concentrations of mixed working solution within the expected range of LODs and LOQs; 2, 3, 4, 6, 8 and 10 ng/50 mg. The samples were extracted and analysed in duplicate for three separate runs. Similarly, LOD and LOQ of 2D GC-NCI-MS for THC-COOH was determined over the range; 10, 20, 30, 40, 50, 60 and 70 pg/50 mg of hair. The concentrations that yielded a reproducible instrument response with  $S/N$  ratio  $\geq 3$  and  $S/N$  ratio  $\geq 10$ , and met all the predefined detection and identification criteria, such as retention time, peak shape, mass spectral ion ratios, were selected as LOD and LOQ, respectively.

#### 4.7.3 Calibration Model

The calibration model can be defined as the mathematical model that demonstrates the relationship between the concentration of analyte and the corresponding instrument response. Establishing calibration model is an obligatory prerequisite for all quantitative methods. The correlation between the signal response (peak area ratio of analyte and internal standard) and analyte concentration in the sample was assessed over a minimum of five replicates per concentration. The concentration range for calibration curves was chosen on the basis of concentrations reported in previous works. For GC-EI-MS, the calibration curve for each analyte was established using zero calibrator, calibrator at LOQs,



20 ng, 50 ng, 100 ng, 200 ng and 300 ng per 50 mg of hair. For 2D GC-NCI-MS, the calibration curve was assessed using the following concentrations; zero calibrator, calibrator at LOQ, 150 pg, 300 pg, 500 pg, 800 pg and 1000 pg/50 mg of hair.

#### 4.7.4 Bias and precision

Bias, which also can be referred to as accuracy or trueness, can be defined as the closeness of agreement between the true value of the analyte concentration and the mean result that is obtained by applying the experimental procedure. Usually, bias is reported as a percent difference. Precision can be defined as the measure of the closeness of agreement between a series of measurements obtained from multiple samplings of the same homogenous sample. For validation of quantitative methods, two different types of precision studies are usually conducted. These two types are within-run precision and between-run precision. It can be called (intra-day) and (inter-day) precisions as well. Bias and precision studies can be carried out concurrently.

For the purpose of calculation of bias and accuracy, a minimum of three separate samples per concentration at two different concentrations were analysed over five different runs. The bias percentage was calculated for each concentration using Equation 4-2:

##### Equation 4-2

Bias (%) at a concentration =

$$\left[ \frac{\text{grand mean of calculated concentration}_x - \text{nominal concentration}_x}{\text{nominal concentration}_x} \right] * 100$$

Using Microsoft excel spreadsheet, both within-run and between-run precisions were calculated using the One-Way Analysis of Variation (ANOVA) approach with the varied factor (run number) as the grouping variable. Using mean square within groups ( $MS_{wg}$ ) and mean square between groups ( $MS_{bg}$ ) obtained from ANOVA table and number of observations in each group (n), within-run CV (%) and between-run CV (%) were calculated using the equations 4-3 and 4-4 below.

**Equation 4-3**

Within-run CV (%) =

$$\left[ \frac{\sqrt{MS_{wg}}}{\text{grand mean for each concentration}} \right] * 100$$

**Equation 4-4**

Between-run CV (%) =

$$\left[ \frac{\sqrt{\frac{MS_{bg} + (n - 1) * MS_{wg}}{n}}}{\text{grand mean for each concentration}} \right] * 100$$

The bias and precision of GC-EI-MS for THC, CBD, CBN and 11-OH-THC, and 2D GC-NCI-MS for THC-COOH were determined by spiking 50 mg of pooled blank hair at two or three quality (QC) levels as shown in Table 4-5. All QC samples were analysed in triplicate over five runs.

**Table 4-5 Summary of QC levels employed for bias and accuracy studies**

Analyte	GC-EI-MS - concentration in ng/mg		
	Low QC	Med QC	High QC
THC	0.2	2	6
CBD	0.2	2	6
CBN	0.2	2	-
11-OH-THC	0.2	2	-
	2D GC-NCI-MS - concentration in pg/mg		
THC-COOH	3	10	16

**4.7.5 Carryover**

Carryover can be defined as the appearance of unintended analyte signal in samples transferred from a previously run positive sample. This signal will, subsequently, lead to inaccurate quantitation. As part of method validation, carryover was evaluated by injecting two blank QC samples immediately after the highest calibration level over five runs whilst establishing the calibration model.

The obtained chromatograms were then examined visually for presence of interfering signal for all analytes.

## 4.8 Method validation results

### 4.8.1 Interference Studies

There were no significant interferences observed from the drug-free hair sources or deuterated ISTD. None of the 91 commonly encountered compounds (shown in Appendix IV) caused interference to signals for the ions monitored for any of the target analytes or internal standards.

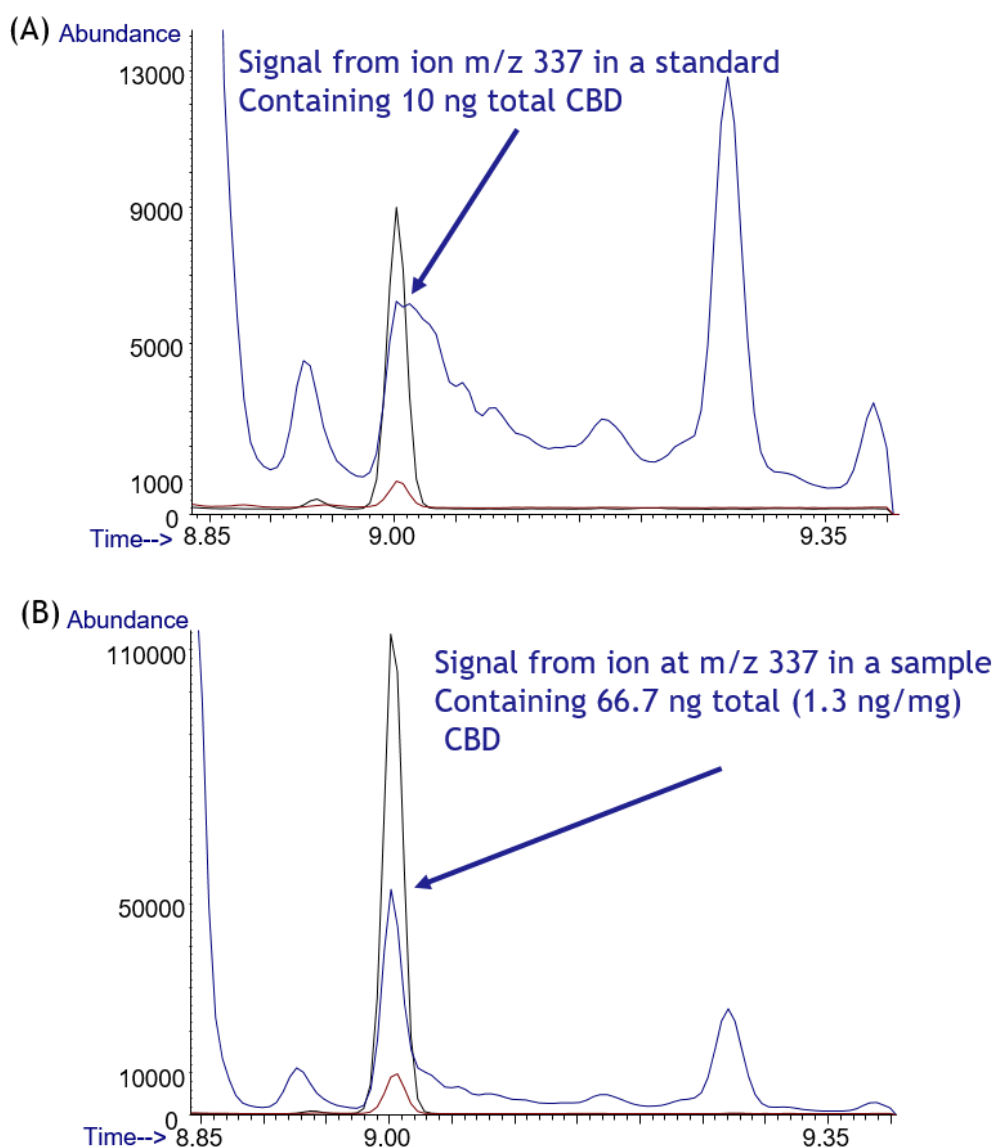
### 4.8.2 Limits of Detection and Limits of Quantification

LOD and LOQ results for all analytes are presented in Table 4-6. LODs for THC, CBD and CBN ranged from 0.04 to 0.12 ng/mg. The achieved LOQ for THC was lower than the cut-off recommended by the Society of Hair Testing (SoHT) ( $\leq 0.1$  ng/mg). The presence of a co-eluting interference at  $m/z$  337 for CBD resulted in elevating its LOD and LOQ to achieve an acceptable ion ratio. Figure 4-8 shows the effect of the interference at  $m/z$  337 in two extraction ion chromatograms from two samples containing different amounts of CBD. To a lesser extent, a small amount of co-eluting interference at  $m/z$  371 has led to a minor distortion of THC ion ratios for the low standards. The SoHT do not have a recommended cut-off for CBD and CBN. Previously published methods for analysis of THC, CBD and CBN in hair matrices have reported limits of detection ranging from 0.0002 to 2.5 ng/mg for THC, 0.005 to 0.9948 ng/mg for CBD and 0.002 to 0.991 ng/mg for CBN (115,124,130,132,133,135,137,138,145,147,153,191).

Despite the fact that 2D GC-NCI-MS showed very good sensitivity for the detection of THC-COOH in unextracted standards (as low as 10 pg total), the detection limit using extracted standards, was higher than this at 50 pg total, equivalent to 1 pg/mg when 50 mg of hair was used. This is higher than the cut-off recommended by SoHT ( $\leq 0.2$  pg/mg). Previously published methods for analysis of THC-COOH in hair matrices have reported limits of detection ranging from 0.01 to 50 pg/mg (137,151,152,155,158,160-163). Extracted chromatograms for the ions of lowest intensity at the LOD and LOQ for all analytes are shown in Figure 4-9.

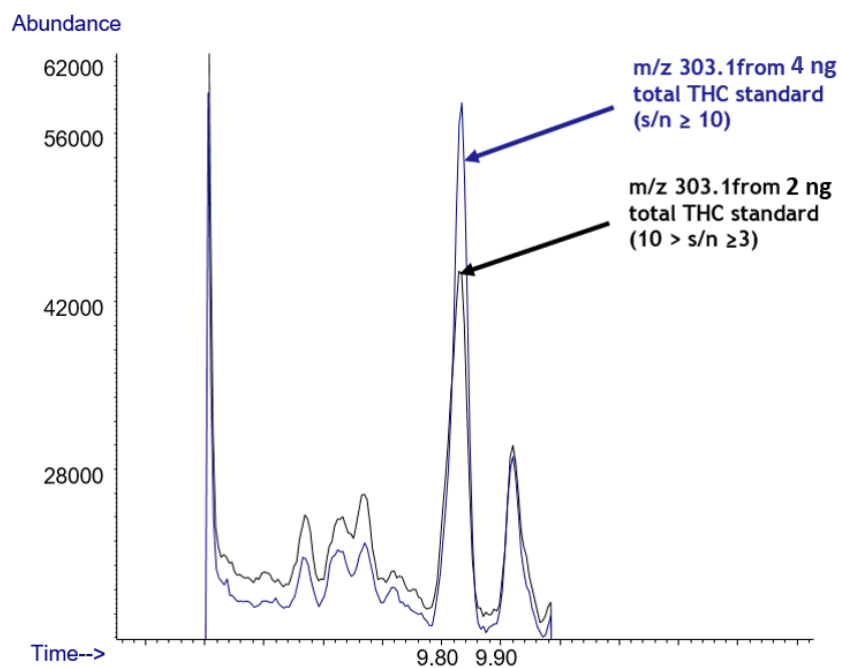
**Table 4-6 Method LOD and LOQ of cannabinoids in hair**

Analytes	GC-El-MS - concentration in ng total (ng/mg)	
	LOD	LOQ
THC	2 (0.04)	4 (0.08)
CBD	8 (0.16)	10 (0.20)
CBN	4 (0.08)	6 (0.12)
11-OH-THC	6 (0.12)	8 (0.16)
	2D GC-NCI-MS - concentration in pg total (pg/mg)	
THC-COOH	30 (0.6)	50 (1)

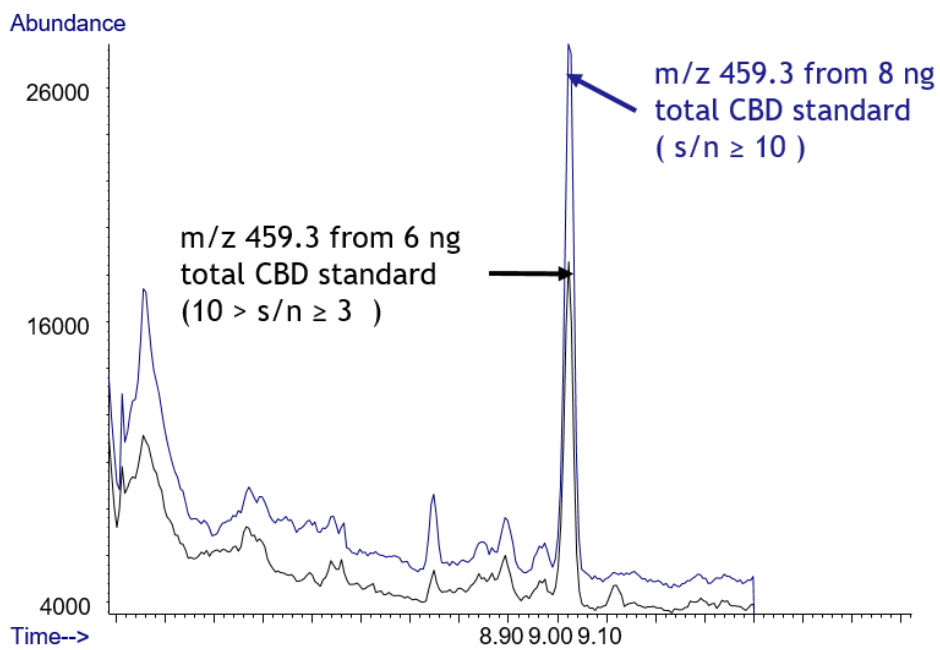


**Figure 4-8** Extracted ion chromatogram CBD identification ions. (A) showing the signal from ion at  $m/z$  337 in a standard containing 10ng total, and (B) sample containing 66.7 ng total.

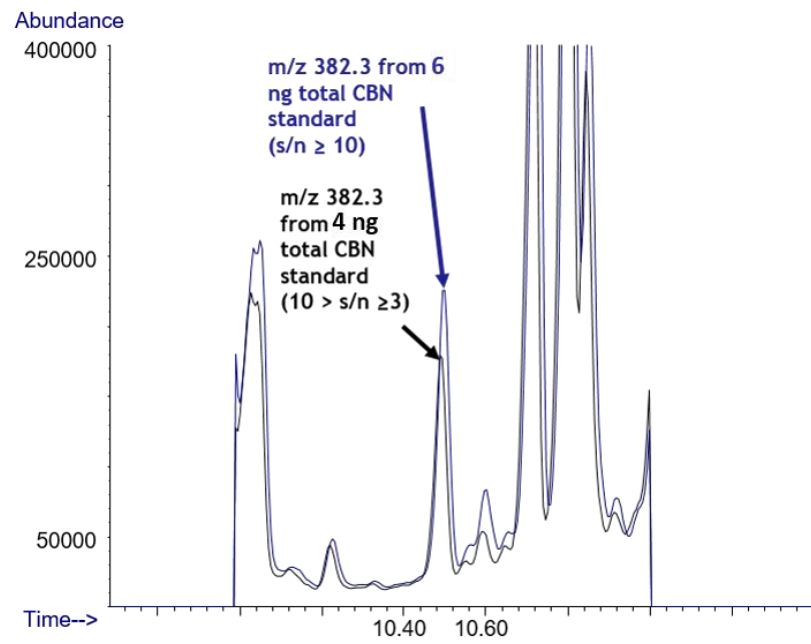
(A)



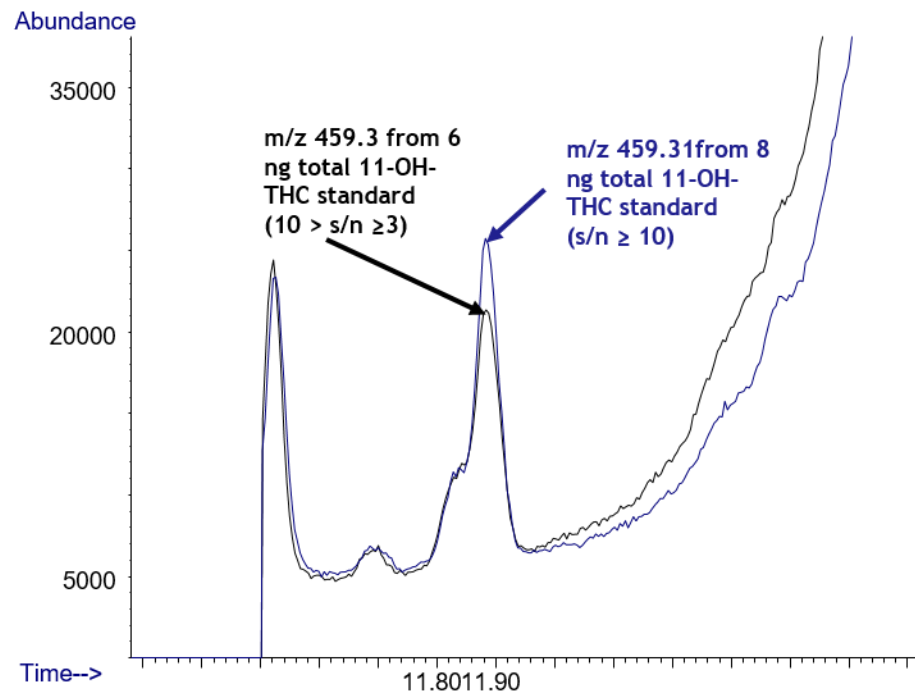
(B)



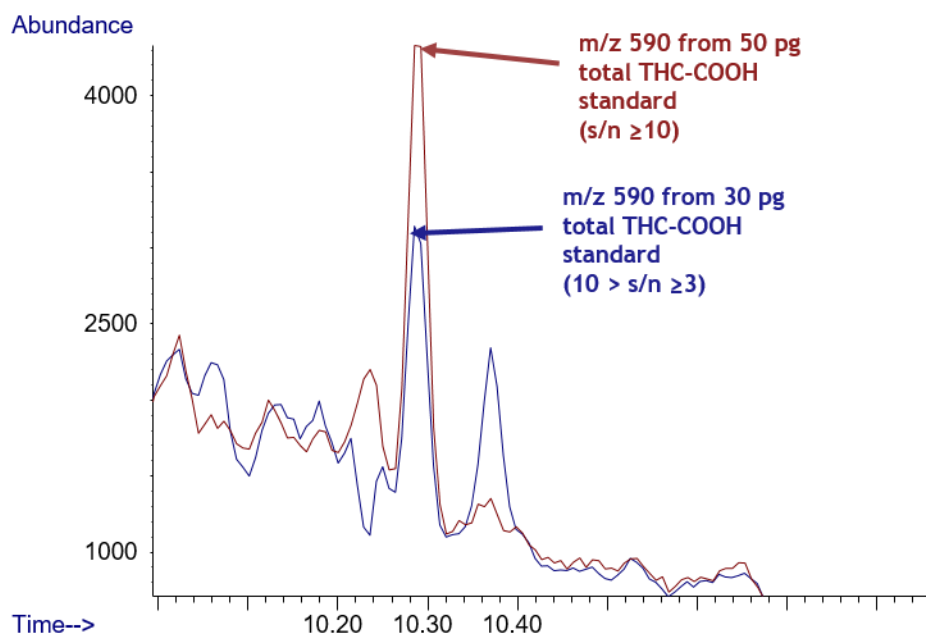
(C)



(D)



(E)



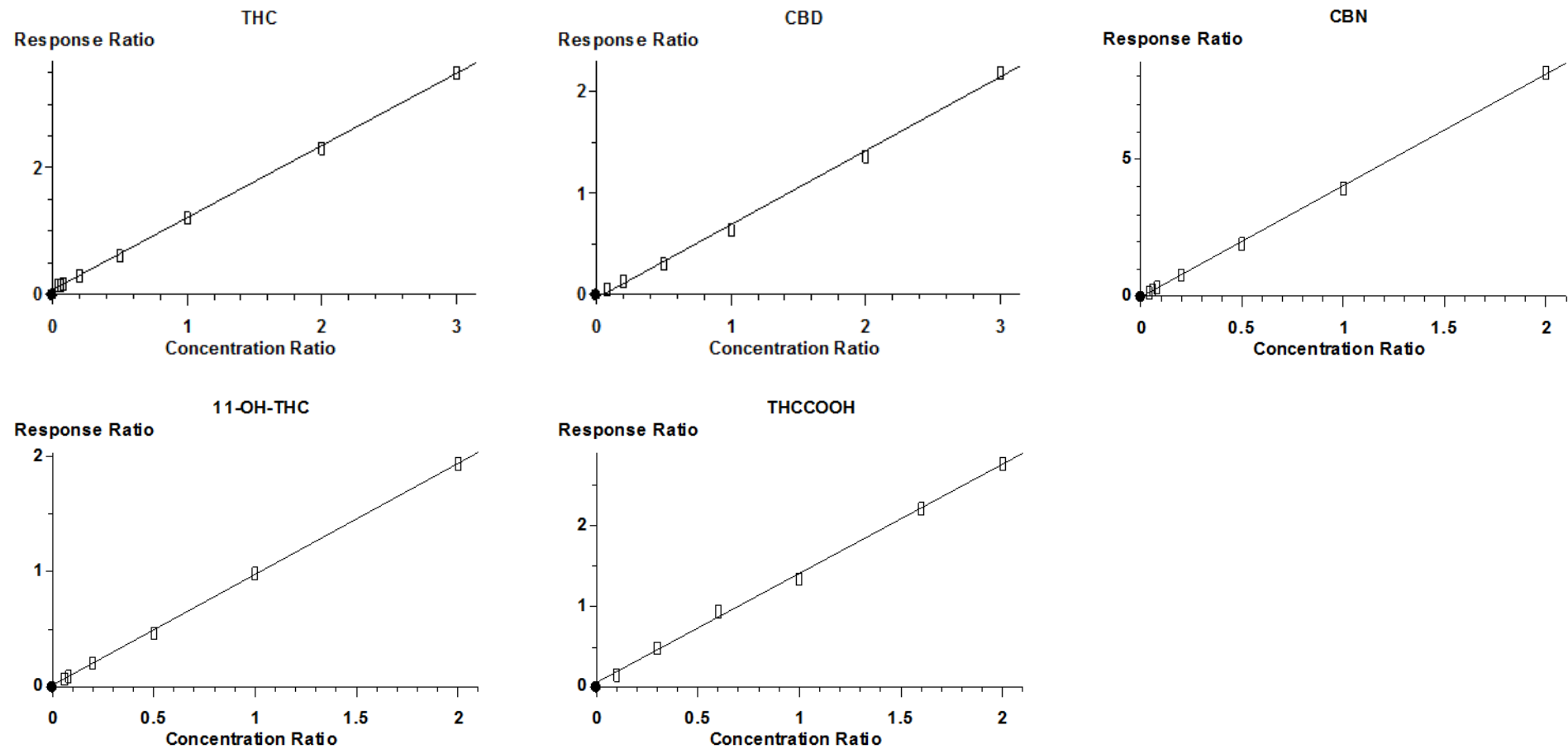
**Figure 4-9** Extracted chromatograms for the ions of lowest intensity at the LOD and LOQ for (A)  $\Delta^9$ -tetrahydrocannabinol (THC), (B) cannabidiol (CBD), (C) cannabinol (CBN) (D), 11-hydroxy- $\Delta^9$ -tetrahydrocannabinol (11-OH-THC), (E) 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH).

### 4.8.3 Calibration Model

The calibration models for all analytes were linear with  $R^2 > 0.99$ . The calibration range for each analyte is shown in Table 4-7. Representative calibration curves for all analytes are shown in Figure 4-10

**Table 4-7** Calibration ranges for all analytes

Analyte	Calibration range
THC	4-300 ng total
CBD	10-300 ng total
CBN	6-200 ng total
11-OH-THC	8 to 200 ng total
THC-COOH	50-1000 pg total



**Figure 4-10** Representative calibration curves for  $\Delta^9$ -tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN) 11-hydroxy- $\Delta^9$ -tetrahydrocannabinol (11-OH-THC), 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) plotted as the total concentration ratio against the response ratio.



#### 4.8.4 Bias and precision

To find out how much of the compound is in the unknown or QC sample, a calibration curve for each compound has been created first using, at least, four levels, and then an aliquot of the case sample to be prepared and analysed in exactly the same way as for the calibration sample. The area of the peak produced by presence of unknown amount of the compound is then compared against the calibration curve to calculate the exact amount of the compound in the sample. Agilent ChemStation software offers different calculation procedures for determining the concentration of each component present in a mixture. Typically, each calculation procedure uses the peak area for the calculation and produces a different type of report. The internal standard (IS) procedure is one of these methods and is accomplished by adding a known amount of a component to both calibration and unknown samples to serve as a normalizing factor. The calibration points are constructed by calculating concentration ratio and a response ratio for each level of a particular peak in the calibration table. The concentration ratio is the amount of the compound divided by the amount of the internal standard at this level. The response ratio is the area of the compound divided by the area of the internal standard at this level. The quantitative results of bias and precision runs for THC, CBD, CBN, 11-OH-THC and THC-COOH are shown in Table 4-8, Table 4-9, Table 4-10, Table 4-11 and Table 4-12. The mean square values, obtained from ANOVA analysis, for within and between groups for THC, CBD, CBN and 11-OH-THC at different QC levels are shown in Table 4-13, and for THC-COOH in Table 4-14. Mean, bias, within and between-run precision results for THC, CBD, CBN and 11-OH-THC are summarised in Table 4-15, and for THC-COOH in Table 4-16.

Table 4-8 Quantitative THC results (ng/mg) of bias and precision runs.

THC					
Low (0.2 ng/mg)	run1	run2	run3	run4	run5
repl.1	0.26	0.25	0.24	0.23	0.26
repl.2	0.27	0.25	0.20	0.19	0.23
repl.3	0.27	0.23	0.18	0.23	0.22
Med (2 ng/mg)	run1	run2	run3	run4	run5
repl.1	2.09	2.01	1.99	2.00	2.06
repl.2	2.15	1.99	1.98	1.98	2.10
repl.3	2.10	2.00	2.00	1.96	2.08
High (6 ng/mg)	run1	run2	run3	run4	run5
repl.1	6.30	5.76	5.17	6.08	5.98
repl.2	5.71	5.75	4.95	6.04	6.09
repl.3	5.41	5.76	5.35	6.07	6.03

Table 4-9 Quantitative CBD results (ng/mg) of bias and precision runs

CBD					
Low (0.2ng/mg)	run1	run2	run3	run4	run5
repl.1	0.24	0.23	0.27	0.24	0.21
repl.2	0.24	0.23	0.26	0.23	0.22
repl.3	0.24	0.24	0.27	0.24	0.23
Med (2 ng/mg)	run1	run2	run3	run4	run5
repl.1	1.58	1.67	1.66	1.80	1.88
repl.2	1.46	1.91	1.90	1.80	1.91
repl.3	1.60	1.99	1.97	1.86	1.85
High (6 ng/mg)	run1	run2	run3	run4	run5
repl.1	5.01	5.97	5.73	6.58	6.33
repl.2	5.10	6.20	6.33	6.40	6.25
repl.3	5.39	5.87	5.62	6.58	6.18

Table 4-10 Quantitative CBN results (ng/mg) of bias and precision runs.

CBN					
Low (0.2ng/mg)	run1	run2	run3	run4	run5
repl.1	0.25	0.23	0.24	0.23	0.20
repl.2	0.25	0.22	0.25	0.23	0.18
repl.3	0.25	0.22	0.24	0.23	0.19
Med (2 ng/mg)	run1	run2	run3	run4	run5
repl.1	1.79	2.01	1.86	2.01	2.17
repl.2	2.04	2.05	1.73	2.01	2.21
repl.3	1.91	1.95	1.68	1.94	2.21

Table 4-11 Quantitative 11-OH-THC results (ng/mg) of bias and precision runs.

11-OH-THC					
Low (0.2 ng/mg)	run1	run2	run3	run4	run5
repl.1	0.21	0.20	0.21	0.21	0.25
repl.2	0.21	0.21	0.21	0.20	0.26
repl.3	0.21	0.22	0.20	0.22	0.25
Med (2 ng/mg)	run1	run2	run3	run4	run5
repl.1	1.90	2.03	1.88	1.89	1.90
repl.2	1.92	2.07	1.88	1.89	1.92
repl.3	1.90	2.07	1.90	1.90	1.93

Table 4-12 Quantitative THC-COOH results (pg/mg) of bias and precision runs.

THC-COOH					
Low (3 pg/mg)	run1	run2	run3	run4	run5
repl.1	3.28	3.04	3.45	3.35	3.38
repl.2	3.36	3.22	3.26	3.47	2.97
repl.3	3.22	3.16	3.48	3.29	3.43
Med (10 pg/mg)	run1	run2	run3	run4	run5
repl.1	8.96	8.96	9.07	9.79	9.24
repl.2	8.94	9.4	9.31	10.18	9.46
repl.3	9.50	9.27	13.55	8.34	9.56
High (16 pg/mg)	run1	run2	run3	run4	run5
repl.1	16.28	15.96	15.72	16.44	15.45
repl.2	14.76	16.08	15.85	15.03	15.99
repl.3	14.60	14.24	15.17	15.61	15.99

Table 4-13 The mean square values for within- and between groups for THC, CBD, CBN and 11-OH-THC at different QC levels.

QC (ng/mg)		THC	CBD	CBN	OH-THC
0.2	BG	0.001716	0.001684	0.001558	0.001185
	WG	0.000421	4.87E-05	2.3E-05	3.55E-05
2	BG	0.011134	0.05732	0.076565	0.014678
	WG	0.000318	0.012382	0.005785	0.000189
6	BG	0.398717	0.774702		
	WG	0.049771	0.045964		

Table 4-14 The mean square values for within- and between groups for THC-COOH at different QC levels.

QC (pg/mg)		THCCOOH
3	BG	0.031388
	WG	0.019708
10	BG	1.138515
	WG	1.496016
16	BG	0.163483
	WG	0.528927

Table 4-15 Summary of mean, bias, within- and between-run precision results for THC, CBD, CBN and 11-OH-THC

THC	Low (0.2 ng/mg)	Med (2 ng/mg)	High (6 ng/mg)
Mean (ng/mg)	0.23	2.03	5.76
Bias (%)	17.42	1.62	-3.93
Within-Run CV (%)	8.74	0.88	3.87
Between-Run CV (%)	12.37	1.24	5.99
CBD	Low (0.2 ng/mg)	Med (2 ng/mg)	High (6 ng/mg)
Mean (ng/mg)	0.24	1.79	5.97
Bias (%)	19.79	-10.51	-0.51
Within-Run CV (%)	2.91	6.22	3.59
Between-Run CV (%)	4.12	8.92	5.98
CBN	Low (0.2 ng/mg)	Med (2 ng/mg)	High (6 ng/mg)
Mean (ng/mg)	0.23	1.97	
Bias (%)	13.78	-1.41	
Within-Run CV (%)	2.11	3.86	
Between-Run CV (%)	2.98	5.56	
11-OH-THC	Low (0.2 ng/mg)	Med (2 ng/mg)	High (6 ng/mg)
Mean (ng/mg)	0.22	1.93	
Bias (%)	8.42	-3.38	
Within-Run CV (%)	2.75	0.71	
Between-Run CV (%)	3.89	1.01	

Table 4-16 Summary of mean, bias, within- and between-run precision results for THC-COOH.

THC-COOH	Low (3 pg/mg)	Med (10 pg/mg)	High (16 pg/mg)
Mean (ng/mg)	3.291	9.569	15.545
Bias (%)	9.708	-4.314	-2.844
Within-Run CV (%)	4.265	12.783	4.679
Between-Run CV (%)	6.079	22.645	6.882

#### 4.8.5 Carryover

No signal was observed in the blank QC samples at the retention time for THC, CBD, CBN, 11-OH-THC and THC-COOH. Carryover was therefore not deemed to be a problem.

### 4.9 Conclusions

The GC-El-MS method was validated for the analysis of THC, CBD, CBN and 11-OH-THC in hair. 2D GC-NCI-MS was validated for analysis of THC-COOH in hair. The two methods validation was carried out for interference, limit of detection (LOD) and limit of quantitation (LOQ), linearity, within and between-day precision, and carryover for all cannabinoids compounds. The developed methods were robust, quick and simple to conduct, and could be integrated into the routine work conducted in the laboratory for hair analysis. Matrix interferences for THC and CBD were encountered. It is believed that the effect of these interferences is limited to cases where THC and CBD are present in low concentrations. Further work on LLE extract cleanliness could eliminate these interferences. In general, the LOQ's were acceptable for all cannabinoids. The LOQ for THC was better than the SOHT recommendation, whereas, the LOQ for THC-COOH was 5 times higher than that recommended by SoHT. Both developed methods were subsequently used in a study involving hair samples collected from known cannabis users in Saudi Arabia, to explore detection rates and concentrations ranges in this part of the world. The results from these analyses are reported and discussed in the next chapter.

## **Chapter 5 Application of the validated methods to analyse cannabinoids in hair of Saudi patients undergoing drug rehabilitation**

### **5.1 Introduction**

Up to this stage, both methods developed for the measurement of cannabinoids in hair samples had only been tested with hair samples spiked with methanolic drug standards. Therefore, it was important to assess its practicality and utility when applied to authentic hair specimens obtained from known cannabis users. In addition, the correlation between the concentrations of each cannabinoid and the self-reported use of cannabis was statistically studied. The detection rate of each cannabinoids was also investigated as the hair samples were collected from an ethnic group that has never been studied. The validity of using a single sample to carry out a liquid-liquid extraction (LLE) followed by solid-phase extraction (SPE) for quantitation of THC-COOH was also investigated.

### **5.2 Ethical approval**

Ethical approval was reviewed and granted by the National Committee of Medical and Bioethics at the Ministry of Health (MOH) in Saudi Arabia, and the Research Ethics Committee within the College of Medical, Veterinary and Life Sciences (MVLS), University of Glasgow. Copies of ethical approval letters from both authorities, the hair collection instructions and consent form are shown in the following appendices Appendix V, Appendix VI, Appendix VII and Appendix VIII.

### **5.3 Authentic hair specimens**

The specimens provided were from cannabis users who had admitted using cannabis and also their urine samples had screened positive for cannabinoids at the time of admission to the addiction hospital (Alamal Hospital, Jeddah, Saudi Arabia). Twenty specimens were collected from the posterior vertex region of male cannabis users with no major hair treatment reported. Each sample was labelled with a unique identifier number at the time of collection. In the laboratory, specimens were stored in a dark and dry place at room temperature until the time of analysis.

The hair colour and length were recorded for each hair specimen at the time of receipt. Hair specimens of more than 3 cm total length were segmented into segments and each segment was placed in a separate 20 mL chromacol environmental vial. The 1st segment representing the most recent growth was 3 cm for all hair specimens except those that were less than 3 cm in total length. A summary of the visual properties of each hair specimen and number of segments are shown in table 5-1.

**Table 5-1 Visual properties of hair specimens and segments**

Sample No.	Hair Colour	Hair Length (cm)	No. of segments
S1	Black	<1	1
S2	Black	4	1
S3	Black	3	1
S4	Black	4	1
S5	Black	6	2
S6	Black	7	2
S7	Black	1.5	1
S8	Black	6	2
S9	Black	7	1
S10	Black	7	1
S11	Black	3	1
S12	Black	16	5
S13	Black	5	1
S14	Black	4	1
S15	Black	3	1
S16	Black	5	1
S17	Black	<1	1
S18	Black	2.5	1
S19	Black	2.5	1
S20	Black	4	1

Participants were interviewed and asked to describe their cannabis use according to the designed questionnaire shown in Appendix IX. Table 5-2 shows a summary of the answers given to questions that provide information regarding (A) the number of joints used per day and (B) the number of days used per week. This



information was collated by Dr Ahmed Alasmari from Ministry of Health, Saudi Arabia under the instruction of Farouq Alzahrani. In the same table, weekly use score (C) was calculated from the provided information in (A) and (B). Use score was calculated by multiplying the minimum and maximum number of joints (cannabis and tobacco) or spliffs (cannabis only) by the minimum and maximum number of days used and this was then averaged. The user who donated sample number 8 (S8) reported a monthly use therefore was assigned zero as the minimum number of days per week to one as the maximum. Cannabis consumers in Saudi Arabia are commonly known to roll joints rather than spliffs, however, the exact method of consumption was not monitored in this study.

**Table 5-2 Summary of answers given to cannabis consumption questions and weekly cannabis use score listed by specimen number (S1 – S20)**

Sample No.	(A) joints/day		(B) days/week		(C) Weekly use score		
	From	To	From	To	From	To	Average
S1	1	3	1	2	1	6	3.5
S2	5	10	3	4	15	40	27.5
S3	2	3	1	2	2	6	4
S4	1	2	7	7	7	14	10.5
S5	2	3	1	2	2	6	4
S6	3	4	3	4	9	16	12.5
S7	20	25	7	7	140	175	157.5
S8	1	2	0	1	0	2	1
S9	1	2	3	4	3	8	5.5
S10	1	2	3	4	3	8	5.5
S11	1	2	1	2	1	4	2.5
S12	1	2	1	2	1	4	2.5
S13	3	4	3	4	9	16	12.5
S14	15	20	7	7	105	140	122.5
S15	4	5	2	3	8	15	11.5
S16	15	20	7	7	105	140	122.5
S17	5	6	7	7	35	42	38.5
S18	20	30	7	7	140	210	175
S19	1	2	3	4	3	8	5.5
S20	1	3	2	3	2	9	5.5

## **5.4 Preparation of hair specimens**

### **5.4.1 Washing the hair specimens**

To remove drug deposited on the outer surface of hair, it was important to solvent wash hair segments. Solvent washes were carried out according to the following protocol and kept for later analysis. 3 mL deionised water were added to each specimen tube containing the hair specimens, sonicated for 3 minutes and transferred to a 7 mL vial for later analysis. This was labelled as water wash (W) followed by specimen number. For instance, the water wash for specimen number one was labelled as W-S1. This was repeated two times with three mL of DCM. The DCM washes were removed into individual clean specimen tubes and labelled as wash DCM 1 and DCM 2 followed by specimen number. To aid the hair drying process, a final rinse of 1 mL of acetone was added to the specimen tubes, swirled around the hair specimens and removed immediately. This rinse was discarded. The hair specimens were left to dry completely overnight.

### **5.4.2 Cutting the hair specimens**

The 20 mL Chromacol environmental vials have a wide open top and allowed for hair cutting to be carried out inside the vials with scissors. Once the hair specimens were completely dry, they were cut as finely as possible into approximately 1-2 mm pieces with sharp scissors to aid the hair digestion process. The scissors were wiped clean with methanol in between the cutting of each hair segment.

### **5.4.3 Weighing out the hair specimens**

Each hair specimen was weighed out into a 7 mL screw cap tube and then the hair decanted into a digestion and extraction tube. It was necessary to weigh samples into the 7 mL vials as the digestion tubes are tapered and will not stand on the analytical balance. In addition, it was found that leaving specimens overnight resulted in statically charged hair samples becoming more neutralised which facilitated dealing with the hair specimens and the accuracy of the hair mass transferred into the digestion tube. The mass of hair specimen varies depending on the amount of hair available for analysis. Most of the specimens were in the range of 30 - 50 mg. The exact amount weighed out was recorded for each

specimen. Approximately 50 mg of drug-free hair was also weighed out for each calibration standard.

#### **5.4.4 Preparation of calibration standards and addition of internal standards**

After the hair was weighed out, a full calibration curve was prepared to run alongside the hair specimens as previously described in Table 4-4 with the inclusion of two additional points as discussed in section 4.7.3. Internal standard solutions (100  $\mu\text{L}$ ) containing 1 ng/ $\mu\text{L}$  THC-*d*3 and 11-OH-THC-*d*3 (equivalent to 2 ng/mg) and (30  $\mu\text{L}$ ) containing 10 pg/ $\mu\text{L}$  THC-COOH-*d*3 (equivalent to 6 pg/mg) were added to each calibration standard and authentic hair specimen with the exception of the blank.

#### **5.4.5 Digestion and extraction**

The digestion and extraction was conducted according to the method that was developed in chapter 4. The final protocols that were followed are shown in figure 5.1.

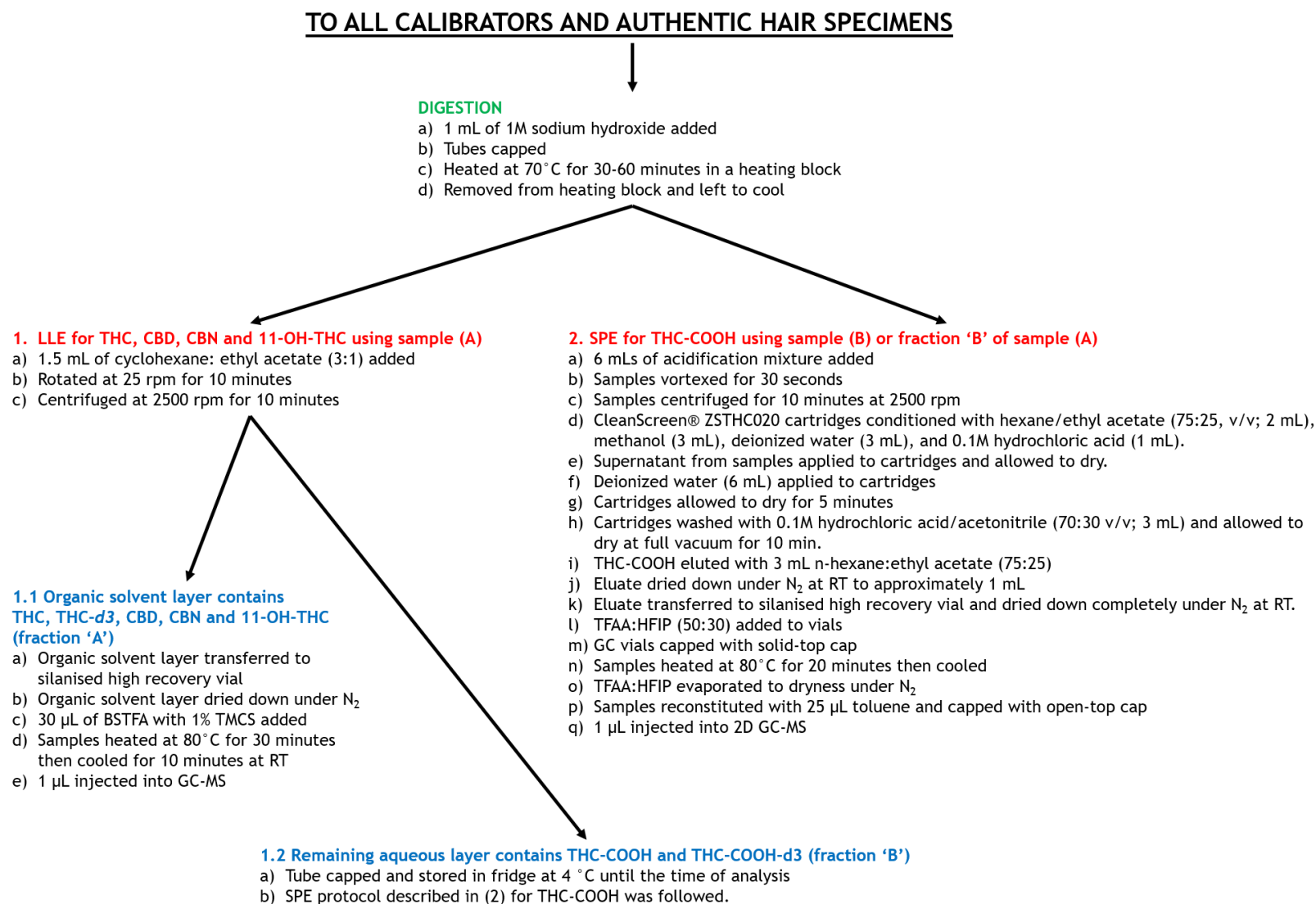
#### **5.4.6 Analysis of the solvent washes**

In routine work, it is common practice to analyse the hair washes if the analysis of the hair specimen produces a positive result to exclude the possibility of external contamination. Hair specimens can be contaminated either due to exposure to cannabis smoke, which increases the concentrations of parent cannabinoids only THC, CBD and CBN, or from secretions of sweat or sebaceous glands surrounding the hair, which may, in addition to parent cannabinoids, increase the concentrations of two main metabolites 11-OH-THC and THC-COOH. In this work, for each hair specimen, one water wash and two solvent washes were produced during the preparation of the hair specimens. These were labelled W, DCM 1 and DCM 2. Prior to analysis of these hair specimens no work had been undertaken to determine the best method of analysis for these washes. If there were cannabinoids present due to contamination from external sources, it would be expected that the concentration of the cannabinoids would be higher in the washes than in the hair itself. It was therefore anticipated that analysis by standard GC-MS for the TMS derivatives of all analytes would be sufficient to

detect the presence of cannabinoids in the washes. Washes were not tested for the presence of THC-COOH.

#### **5.4.7 Preparation of solvent washes for analysis**

All washes were stored in fridge at 4 °C. At the day of analysis, washes were left to dry overnight in the fume hood. Once samples were completely dry, they were reconstituted with 1 ml of 1M NaOH and 100 ng (100 µl of 1 µg/mL internal standard solution mixture containing THC-d3 and 11-OH-THC-d3) was added. Washes were then subjected to the LLE method developed for hair samples as shown in points 1 and 1.1 in Figure 5-1.



**Figure 5-1 Protocol for digestion and extraction of hair specimen**

## 5.5 Qualitative identification of analytes

### 5.5.1 THC/CBD/CBN/11-OH-THC

Mass spectrometry equipped with an electron ionisation (EI) source was operated in selective ion monitoring (SIM) mode, monitoring ions were:  $m/z$  371, 386, 303 for THC-TMS; 390, 337, 458 for CBD-di-TMS; 367, 368, 382 for CBN-TMS, 371, 474, 459 for 11-OH-THC di-TMS, and 374, 389 for THC-TMS- $d_3$  as internal standard for THC, CBD and CBN, and 374, 462 for 11-OH-THC di-TMS- $d_3$  as internal standard for 11-OH-THC. Underlined ions were used as the quantifying ions, and the others used as qualifiers. Each analyte was identified and confirmed positive by the presence of its three SIM ions in the mass spectrum at the correct RT ( $\pm 0.15$  min) and in the correct ratio ( $\pm 20\%$  of the calibrator ion ratio) (192).

### 5.5.2 THC-COOH

Mass spectrometry equipped with negative chemical ionisation (CI) source was operated in selective ion monitoring (SIM) mode, monitoring ions: 422, 590 for THC-COOH TFAA/HFIP derivative; and 425, 593 for THC-COOH- $d_3$  derivative as internal standard for THC-COOH. Underlined ions were used as the quantifying ions, and the others used as qualifiers. As NCI is a softer ionization technique than EI, positive identification of THC-COOH was different to the other analytes. Positive identification was based on the criteria set by Moore *et al* with similar techniques (141,152). Moore *et al* stated that:

1. The selected SIM ions in the mass spectrum should be present within 30% of the calibration ratio.
2. The peak should be present at the correct retention time compared to the internal standard.
3. Should be compared to known controls/calibrators in the same batch
4. There should also be satisfactory chromatographic removal of other peaks arising from the matrix.

### 5.5.3 Calculation of concentrations in authentic specimens

A case sample is a sample containing an unknown amount of the compound to be quantified. To find out how much of the compound is in the unknown sample, the method described previously in section 4.8.4 for the quantitation of validation QC samples was followed. To simplify calculations and allow for varying hair mass, the calibration curves for each analyte were constructed as ng total (for THC, CBD, CBN and 11- OH-THC) and pg total (for THC-COOH) rather than as ng/mg and pg/mg. When an analyte had been identified as present qualitatively, the peak area of the analyte and internal standard was calculated by the Agilent ChemStation software package. Integration of extracted ion chromatograms for each calibrator, QC, sample and case sample were reviewed and adjusted, if needed, using the QEdit tool on ChemStation. The software takes the appropriate response factors obtained from a previous calibration stored in the method. Using the internal standard concentration and peak areas from the run, the software calculates component concentrations. This gave a calculated value for the total concentration of drug in the whole weight of the hair sample. The concentration of analyte per mg of hair was then calculated.

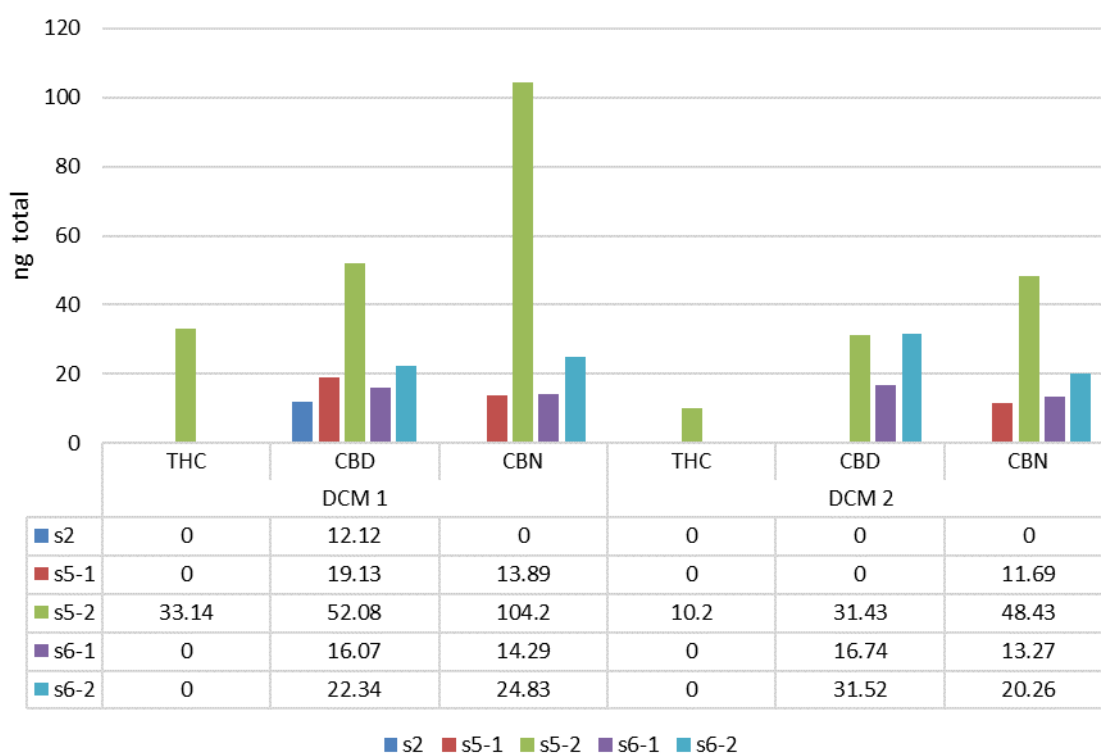
## 5.6 Statistical Analysis

Statistical analysis was carried out using IBM SPSS Statistics Version 22 on the concentrations of THC, CBD, CBN and THC-COOH detected in the first segments only of hair specimens. For THC-COOH, concentrations resulting from the analysis of sample (B) were used for statistical analysis. As the data was found not to follow a normal distribution, non-parametric tests were employed. The Mann Whitney U test was used to assess if there was any significant difference in distribution of the concentrations of THC, CBD, CBN and THC-COOH detected between non-daily and daily users. Spearman's Rank Correlation Test (1-tailed) was used to assess whether the concentrations of THC, CBD, CBN and THC-COOH correlated with the number of joints smoked by each user. A p value < 0.05 was considered significant.

## 5.7 Results from analysis of authentic hair specimens

### 5.7.1 Results of analysis of the washes

There was no 11-OH-THC detected in any of the washes. Also, none of the analytes were detected in any of the water washes. In the first DCM wash (DCM1), THC was detected for one case (S5-2), CBD and CBN were detected in the washes of five cases (S2, S5-1, S-52, S6-1, S6-2) and four cases (S5-1, S-52, S6-1, S6-2), respectively. In the second DCM wash (DCM2) for S5-2, THC was detected in the same wash but with approximately 70% decrease in concentration. CBD concentrations fell below the detection limit in two out of the five samples (S2, S5-1) for DCM2. No significant decrease was observed in the concentrations from washes of the other three samples. CBN was detectable in the second wash of all four samples, and with the exception of S5-2, there was no significant decrease in the concentrations of CBN between the two washes. The concentrations of the cannabinoids detected in the DCM washes of each hair specimen are summarised in Figure 5-2.



**Figure 5-2** The concentrations of  $\Delta^9$ -tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN) (ng total) detected in the DCM washes of each hair specimen. Specimens that are not included did not have positive washes for any cannabinoid.



### 5.7.2 Concentrations detected in authentic hair specimens

Of the 20 hair specimens (27 segments) analysed, only eight contained no detectable cannabinoids or metabolites. All specimens were negative for 11-OH-THC as expected. THC, CBD and CBN were detected in 4, 14 and 4 specimens respectively. THC-COOH was detected in 8 specimens, when specimen (A) was re-extracted, and in 15 specimens when sample (B) was tested. A summary of the range, mean and median concentrations detected for each analyte are shown in Table 5-3. The concentrations of the cannabinoids detected in each hair specimen are shown in Table 5-4, and Table 5-5. Figure 5-3, Figure 5-4, Figure 5-5, and Figure 5-6 display the extracted ion chromatograms for a hair specimen (S5-1) that was positive for THC, CBD, CBN and has the second highest THC-COOH concentration. For comparison, the extracted blank and two lowest extracted standards for each analyte are also shown.

**Table 5-3 Descriptive statistics of the concentrations detected for  $\Delta^9$ -tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN) and 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in 27 authentic hair specimens from cannabis users.**

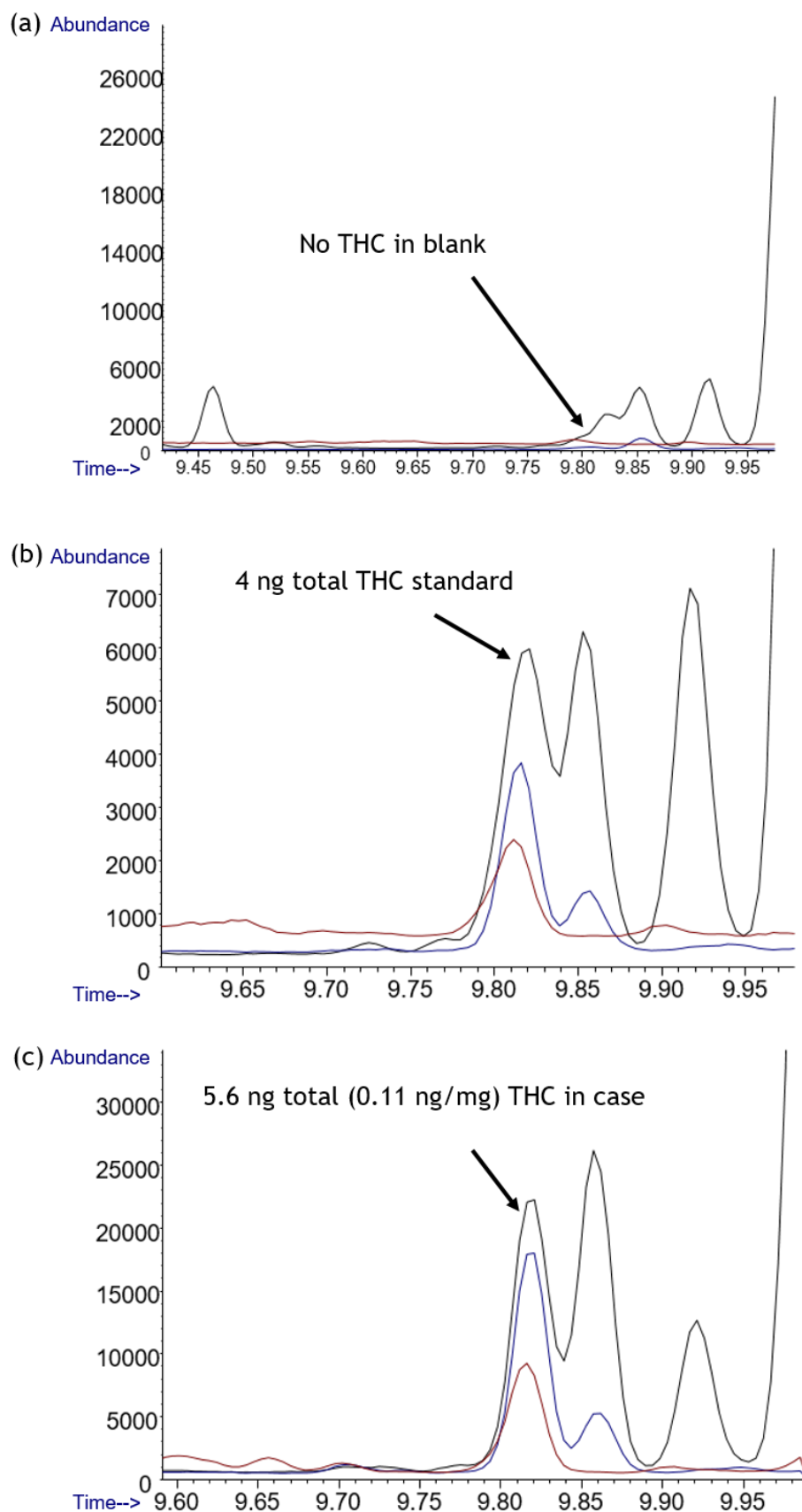
	THC (ng/mg)	CBD (ng/mg)	CBN (ng/mg)	THC-COOH sample (A) (pg/mg)	THC-COOH sample (B) (pg/mg)
Mean	0.17	1.07	0.53	2.71	2.58
Median	0.11	0.45	0.40	2.65	2.14
Minimum	0.11	0.20	0.31	1.05	0.93
Maximum	0.34	4.42	1.02	5.53	7.01
No. positive	4	14	4	8	15

**Table 5-4 Concentrations of  $\Delta^9$ -tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN) in each hair specimen**

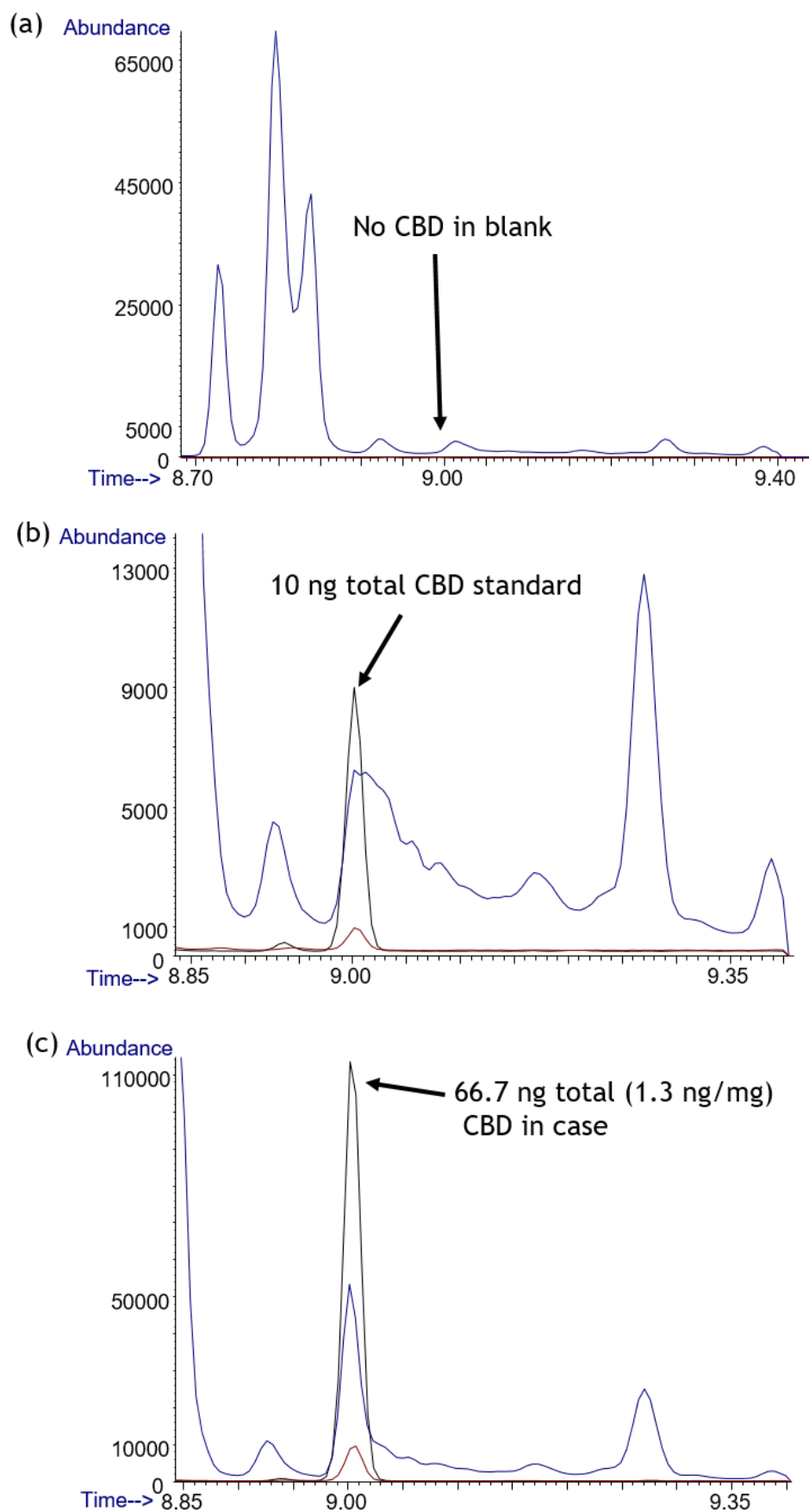
Sample ID	Weight (mg)	Drug concentrations (ng/mg)		
		THC	CBD	CBN
s1	29.6	N.D	0.29	N.D
s2	55.7	N.D	0.40	N.D
s3	52.2	N.D	N.D	N.D
s4	50.5	N.D	N.D	N.D
s5-1	51.3	0.11	1.30	0.33
s5-2	50.2	0.34	4.42	1.02
s6-1	50.1	N.D	1.41	0.31
s6-2	50.0	0.11	2.24	0.48
s7	47.1	N.D	N.D	N.D
s8-1	50.2	0.12	1.99	N.D
s8-2	49.2	N.D	N.D	N.D
s9	50.5	N.D	N.D	N.D
s10	49.3	N.D	N.D	N.D
s11	50.4	N.D	N.D	N.D
s12-1	48.2	N.D	N.D	N.D
s12-2	48.7	N.D	N.D	N.D
s12-3	51.5	N.D	0.21	N.D
s12-4	49.1	N.D	0.37	N.D
s12-5	50.2	N.D	0.50	N.D
s13	51.5	N.D	0.20	N.D
s14	49.9	N.D	0.21	N.D
s15	53.3	N.D	N.D	N.D
s16	50.1	N.D	0.23	N.D
s17	52.2	N.D	N.D	N.D
s18	49.9	N.D	N.D	N.D
s19	50.4	N.D	N.D	N.D
s20	34.4	N.D	1.22	N.D

**Table 5-5 Concentrations of 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in each hair specimen (sample A and B for each subject).**

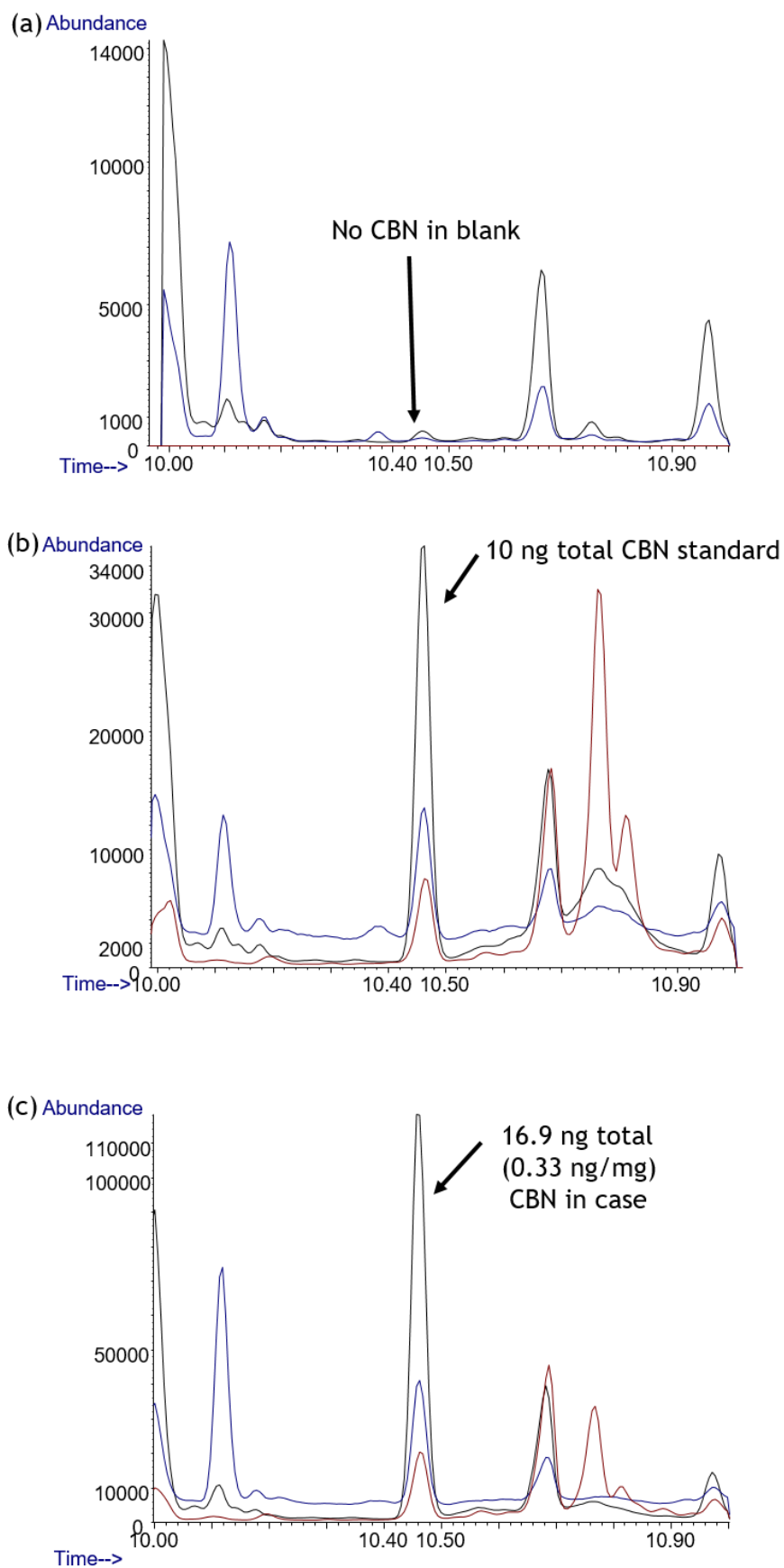
sample ID	LLE followed by SPE for fraction 'B' of sample (A)		SPE for sample (B)	
	weight (mg)	THCCOOH (pg/mg)	weight (mg)	THCCOOH (pg/mg)
s1	29.6	3.22	55.1	2.48
s2	55.7	1.20	50.7	2.33
s3	52.2	N.D	50.4	0.93
s4	50.5	N.D	51.9	N.D
s5-1	51.3	4.18	35.4	4.80
s5-2	50.2	2.41	49.7	3.49
s6-1	50.1	1.05	55.6	2.68
s6-2	50.0	N.D	50.5	1.89
s7	47.1	5.53	29.5	7.01
s8-1	50.2	N.D	53.1	N.D
s8-2	49.2	N.D	54.9	N.D
s9	50.5	N.D	48.3	1.32
s10	49.3	N.D	50.1	N.D
s11	50.4	N.D	51.8	N.D
s12-1	48.2	N.D	48.2	N.D
s12-2	48.7	N.D	50.3	N.D
s12-3	51.5	N.D	51.9	N.D
s12-4	49.1	1.18	49.1	N.D
s12-5	50.2	N.D	41.5	N.D
s13	51.5	N.D	49.1	N.D
s14	49.9	N.D	52.0	1.82
s15	53.3	N.D	49.8	2.14
s16	50.1	N.D	52.2	2.06
s17	52.2	N.D	50.4	1.54
s18	49.9	N.D	26.4	N.D
s19	50.4	N.D	52.3	2.43
s20	34.4	2.89	43.7	1.79



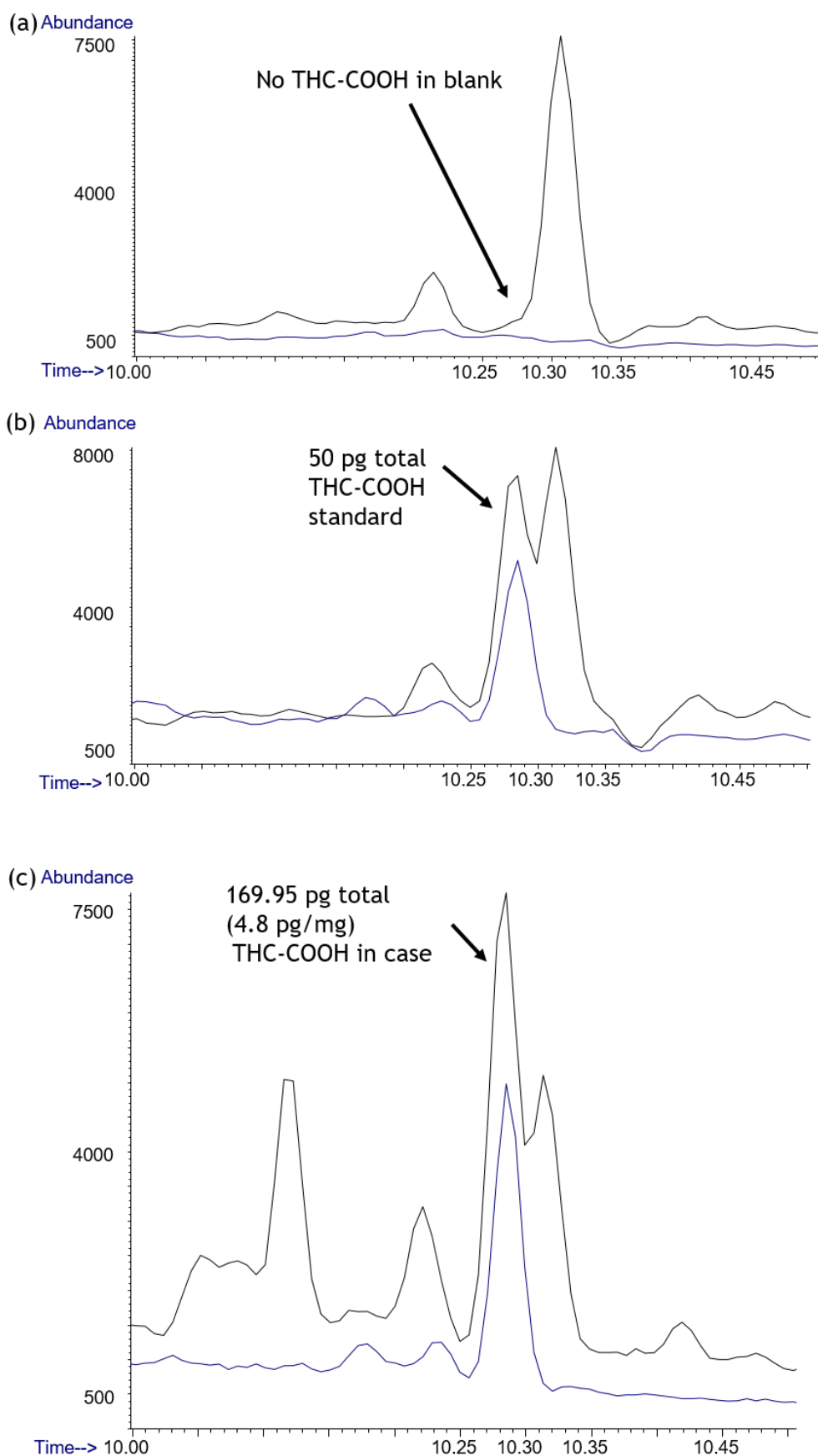
**Figure 5-3** Extracted ion chromatograms for  $\Delta^9$ -tetrahydrocannabinol for (a) an extracted blank (b) an extracted standard containing 4 ng total THC and (c) cannabis positive hair specimen S5-1 (51.3 mg) containing 0.11 ng/mg THC.



**Figure 5-4** Extracted ion chromatograms for cannabidiol (CBD) for (a) an extracted blank (b) an extracted standard containing 10 ng total CBD (c) cannabis positive hair specimen S5-1 (51.3 mg) containing 1.3 ng/mg CBD



**Figure 5-5** Extracted ion chromatograms for cannabidiol (CBN) for (a) an extracted blank (b) an extracted standard containing 6 ng total CBN (c) cannabis positive hair specimen S5-1 (51.3 mg) containing 0.33 ng/mg CBN



**Figure 5-6** Extracted ion chromatograms for 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) for (a) an extracted blank (b) an extracted standard containing 50 pg total THC-COOH (c) cannabis positive hair specimen S5-1 (51.3 mg) containing 4.17 pg/mg THC-COOH.

Table 5-6 summarises the interpretation of results. The interpretation was carried out based on the fact that the presence of parent cannabinoids (THC, CBD and CBN) exclusively will prove exposure to cannabis only, while detecting a metabolite will prove ingestion. Absence of all cannabinoids was interpreted as no exposure or ingestion. Each (3 cm) segment represents the history of the corresponding three months. Sample s12 was segmented to 5 segments. Only the fourth and fifth segments were tested positive for CBD. These segments correspond to the past 6-9 months and 9-12 months. Therefore, result interpretation was categorised as exposure to cannabis in the past year.

**Table 5-6 A summary of the interpretation of the results.**

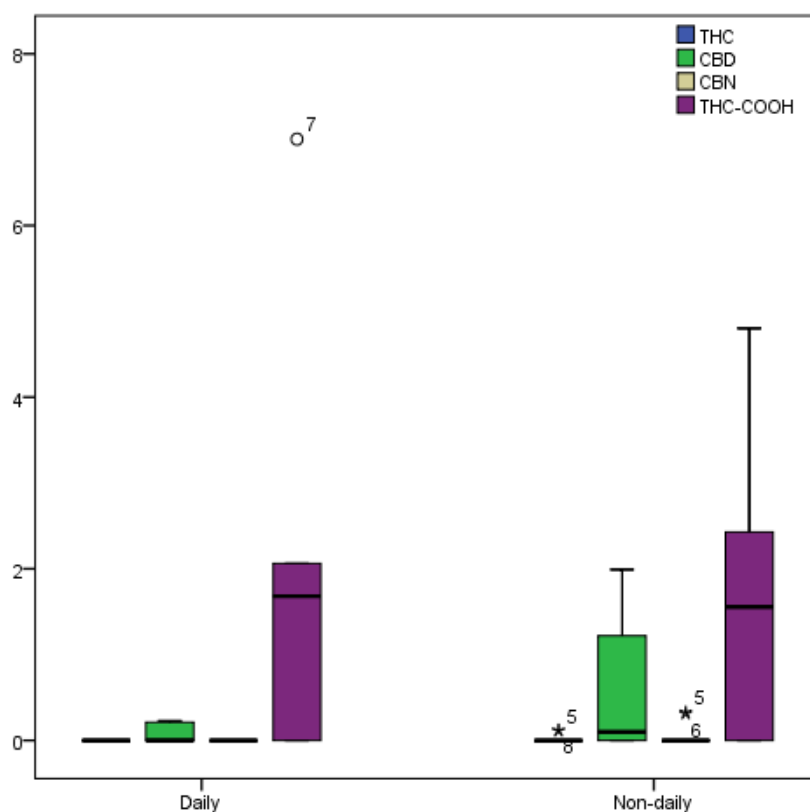
	THC	CBD	CBN	11-OH-THC	THCCOOH	Interpretation
s1	-		-	-	+	Ingestion in past 3 months
s2	-		-	-	+	
s3	-	-	-	-	+	
s5-1	+	+	+	-	+	
s5-2	+	+	+	-	+	
s6-1	-	+	+	-	+	
s6-2	+	+	+	-	+	
s7	-	-	-	-	+	
s9	-	-	-	-	+	
s14	-	+	-	-	+	
s15	-	+	-	-	+	
s16	-	+	-	-	+	
s17	-	-	-	-	+	
s19	-	-	-	-	+	
s20	-	+	-	-	+	
s8-1	+		-	-	-	Exposure in past 3 months
s8-2	-	-	-	-	-	
s10	-	+	-	-	-	
s13	-	+	-	-	-	
s12-1	-	-	-	-	-	Exposure in past year
s12-2	-	-	-	-	-	
s12-3	-	+	-	-	-	
s12-4	-	+	-	-	-	
s12-5	-	+	-	-	-	
s4	-	-	-	-	-	No exposure/ingestion in past 3 months
s11	-	-	-	-	-	
s18	-	-	-	-	-	



### 5.7.3 Correlation of results with cannabis score assigned to each hair specimen

#### 5.7.3.1 Type of user

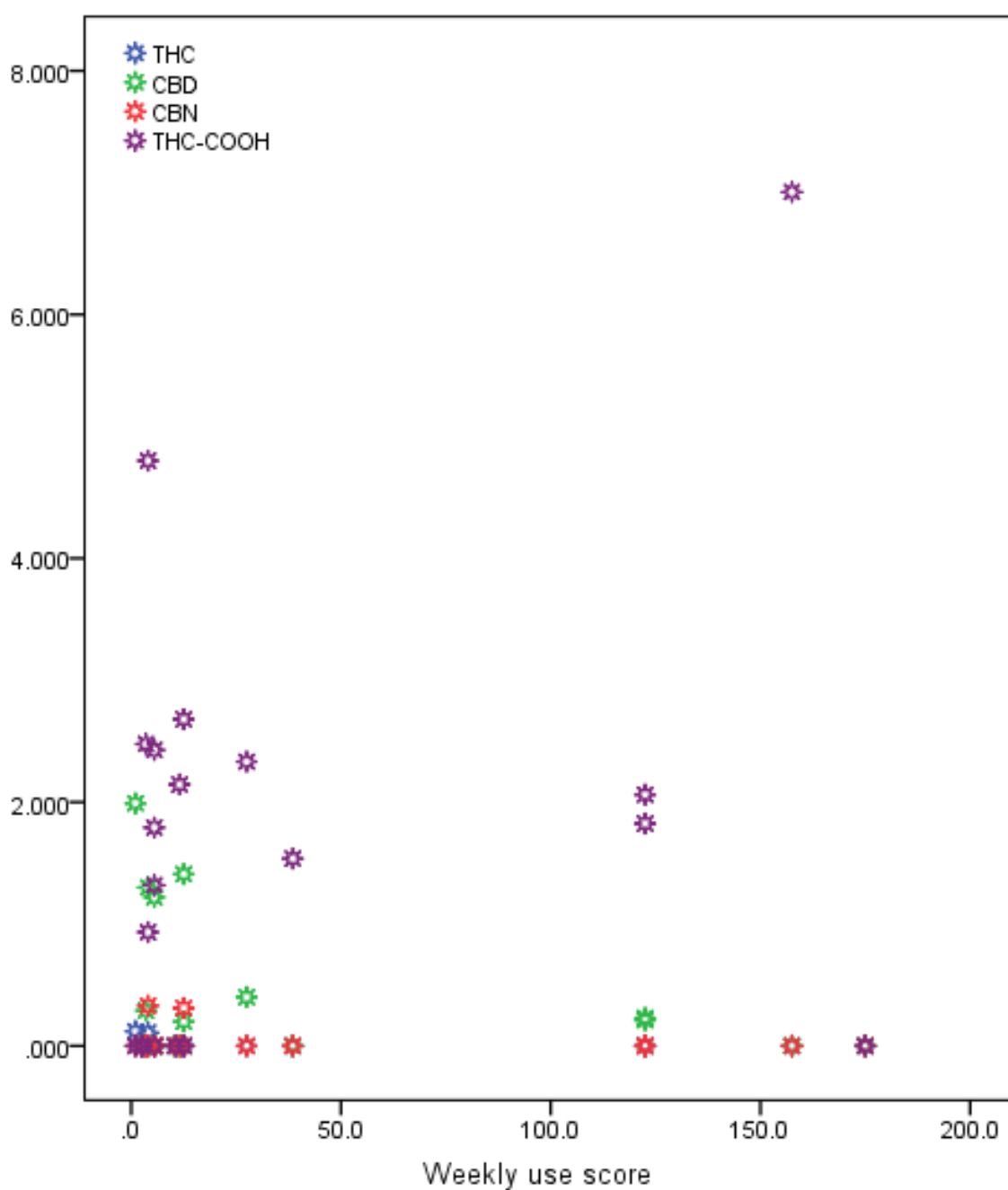
From the information provided on the interview questionnaire, all users, except user number 8, described their cannabis use as either one or more times a week or daily cannabis use. Donor number 8 claimed monthly use. Therefore, samples were grouped into either daily or non-daily users. There were 14 Non-daily users and 6 daily cannabis users. It was found that there was no difference in the distribution of concentrations observed for both types of users for THC, CBD, CBN and THC-COOH. It is noteworthy that the highest THC-COOH concentration was quantified from a daily user's hair sample. The box-plots for each group are shown in Figure 5-7.



**Figure 5-7** Boxplot diagram representing the median and interquartile range of concentrations detected in daily and non-daily cannabis users for  $\Delta^9$ -tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN) (ng/mg) and 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) (pg/mg). There was no significant difference ( $p>0.05$ ) in the distribution of concentrations detected between daily and non-daily users.

### 5.7.3.2 Weekly use score

A weekly use score was calculated based on self-report information given by each user. During the interview, users were asked questions about their weekly cannabis use behaviour during the previous three months. An average weekly score was assigned based on how many days per week they smoked cannabis and how many joints or spliffs they smoked on a day in a week. The ranges of two values were multiplied to provide the range provided for the joints or spliffs smoked per week. Highest and lowest values were then averaged to give an estimation of weekly use score. As all donors reported a constant use pattern in the previous three months, the weekly use score was accepted to represent the 3 months' use. Concentrations of analytes in the first segments only, which represent the cannabis consumption in no more than three months, were included in the statistical analysis. The data was examined to determine if there was any correlation between weekly use score and the concentrations of each cannabinoid and metabolite detected. It was assumed that there would be an increase in concentrations as the weekly use score increased. It was found that there was no correlation between the weekly use score and the THC, CBD, CBN and THC-COOH concentration when examined individually or when both type of users (daily and non-daily) were compared. The scatter plot diagram for all analytes compared to the number of joints smoked is shown in Figure 5-8.



**Figure 5-8 Scatter plot diagram showing no correlation between the weekly use score and the concentrations of  $\Delta^9$ -tetrahydrocannabinol (THC) ng/mg (Spearman  $\rho = 0.416$ ,  $p > 0.05$ ,  $R^2 0.042$ ) cannabidiol (CBD) ng/mg (Spearman  $\rho = 0.416$ ,  $p > 0.05$ ,  $R^2 0.064$ ) cannabinol (CBN) ng/mg (Spearman  $\rho = 0.416$ ,  $p > 0.05$ ,  $R^2 0.029$ ) and 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) pg/mg (Spearman  $\rho = 0.176$ ,  $p > 0.05$ ,  $R^2 0.097$ ) detected in authentic hair specimens.**

## 5.8 Discussion of results

### 5.8.1 Detection rate of different cannabinoids in case specimens

Four (20%) of the 20 hair specimens had no detectable  $\Delta^9$ -THC, CBD, CBN, or THC-COOH at or above the described LOQs. THC-COOH was present in more hair specimens than  $\Delta^9$ -THC with 7 specimens (35%) having only THC-COOH, one (5%) only  $\Delta^9$ -THC and 3 (11.1%) with both. CBD had a high detection rate with 8 positive samples (40%). CBN was detected in 2 (10%) hair samples. The main metabolite THC-COOH had the highest detection rate of all cannabinoids and was detected in 13 (65%) hair samples. Table 5-7 summarises the numbers and percentages of positive and negative hair specimens for each analyte in both daily, non-daily user and both groups combined. Table 5-8 summarises the numbers and percentages of positively identified analytes in all 20 cases.

**Table 5-7 Numbers and percentages of negative and positive samples for  $\Delta^9$ -tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN) and 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in daily, non-daily and both type of users combined.**

	Daily users		Non-Daily users		All users	
	Positive	Negative	Positive	Negative	Positive	Negative
THC	0 (0%)	6(100%)	2 (14.3%)	12 (85.7%)	2 (10%)	18 (90%)
CBD	2 (33.3%)	4 (66.7%)	7 (50%)	7 (50%)	9 (45%)	11 (90%)
CBN	0 (0%)	6 (100%)	2 (14.3%)	12 (85.7%)	2 (10%)	18 (90%)
THC-COOH	4 (66.7%)	2 (33.3%)	9 (64.3%)	5 (35.7%)	13 (65%)	7 (35%)

**Table 5-8 Qualitative results from research of  $\Delta^9$ -tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN) and 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in 20 hair samples**

	Number of sample	Percentage %
None	4	20
THC only	1	5
CBD only	2	10
THCCOOH only	7	35
CBD, THC-COOH	4	20
CBD, CBN, THC-COOH	1	5
THC, CBD, CBN, THC-COOH	1	5

### 5.8.2 Validity of using two extraction procedures on the same hair sample for THC-COOH quantitation

Typically, drug testing in biological samples goes through two stages, screening and confirmation. It is also possible that analysis might need to be repeated for several reasons. Insufficient hair mass is one of the problems that is usually encountered in hair testing. In this thesis, a method to analyse THC-COOH in situations where a limited amount of sample is available is proposed. The validity of retaining the aqueous layer of sample (A) after carrying out a liquid-liquid extraction for THC, CBD, CBN and 11-OH-THC and re-extracting later using solid-phase extraction for THC-COOH was investigated. After carrying out the LLE, vials that contained the aqueous layer were capped and stored in the fridge at 4°C. As the aim of this study was to monitor the changes in concentrations, internal standard for THC-COOH was not added until the time of SPE extraction. The obtained concentrations after analysis were compared against those quantified in sample (B) which was extracted using SPE only. Figure 5-9 shows a comparison of concentrations of samples A and B for each subject. Negative samples in both procedures were excluded from the bar chart. 16 samples were tested positive for at least one extraction procedure. Half of the samples (n=8) were tested positive only in sample (B). 5 samples had higher concentrations in sample (B) than sample (A) with a mean % increase of 22.5% (7-43%). The remaining three samples were found to have higher concentrations in sample (A). Two of these three samples were found to have higher concentrations in samples (A) than sample (B) with a mean % increase of 18.3% (13-23.6%), and one sample was positive only in sample (A). To conclude, it is believed that retaining the LLE aqueous layer for further extraction is an acceptable approach when there is insufficient sample for further analysis. The noticed decrease in concentration could be due to loss of analyte with LLE extraction or a stability issue during storage between the two analyses or both. Adding IS for THCCOOH analysis earlier will correct the decrease in concentration but will not change the rate of false negatives.

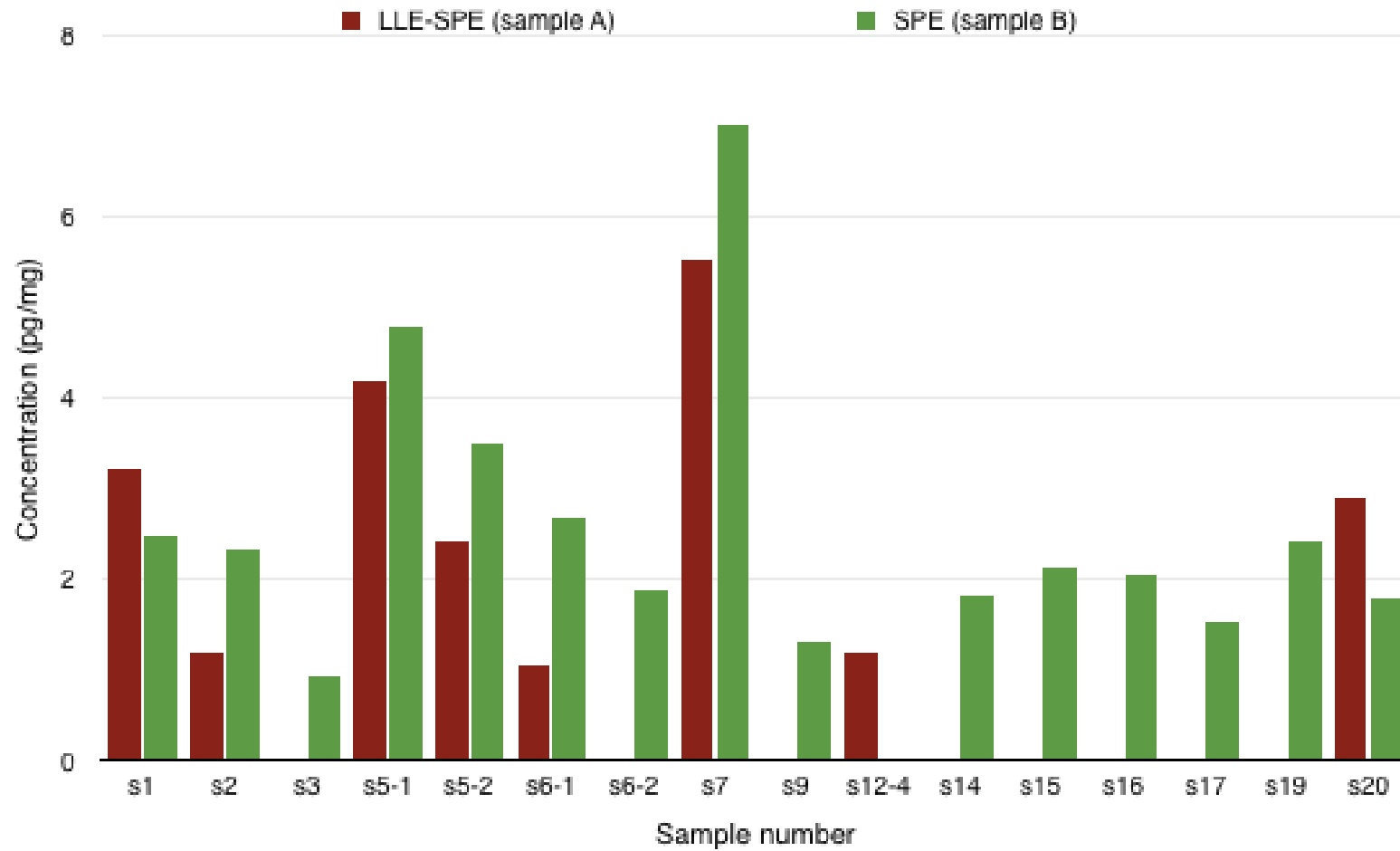


Figure 5-9 Concentrations of 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) quantified in sample (A) after LLE followed by SPE and in sample (B) after SPE.

### 5.8.3 Comparison of results to other studies

There have been many studies published in the literature looking at the concentrations of different cannabinoids in hair. The targeted analytes vary between studies. The majority have focussed on measuring only the main metabolite THC-COOH, as it is believed to, at least, minimise claims of external exposure, whilst others have only measured the major parent cannabinoids (THC, CBD and CBN), which can only confirm exposure to cannabis. Simultaneous analysis for the main psychoactive cannabinoid (THC) and its main metabolite was found popular in the literature as well. Only four studies have determined concentrations for all four compounds. Table 5-9 lists the references by the targeted analyte(s) and the number of studies where they were analysed.

**Table 5-9 Classification of cannabis in hair publications based on targeted analyte(s).**

Targeted analytes	n	References
THC-COOH only	15	(123,139,141,143,151,152,154-156,158,160-163,193)
THC, CBD & CBN	12	(124-127,132,133,135,136,144,145,148,159)
THC & THC-COOH	9	(128,134,137,138,140,142,147,153,194)
THC only	7	(116,121,122,129,191,195,196)
THC, CBD, CBN & THC-COOH	4	(115,120,130,131)
THC, CBN & THCA A	2	(117,118)
THC & THCA A	2	(65,146)
THC, THC-COOH & 11-OH-THC	2	(150,167)
THC, CBD, CBN & THCA A	1	(197)
THC, CBN & THC-COOH	1	(114)
11-OH-THC	1	(198)
THC, THC-COOH & THC-COOglu	1	(108)
THC, THC-OOH, Alcohol & THC-COOEt	1	(149)
<b>Total</b>	<b>58</b>	

In the following sections (5.7.3.1 - 5.7.3.4), the concentrations detected in this study (shown in tables 5.4 and 5.6 (SPE only)) have been compared to those reported in the previous studies. Based on the above table 5-9, the number of studies that targeted THC, CBD, CBN and THC-COOH in hair specimens in the literature are 43, 18, 21 and 43, respectively. Several studies have been excluded from comparison. Examples of these are studies which focussed mainly on method development and validation where no case samples, or only proficiency test samples were tested (65,120,122,151,195), or only one case sample (121,167), or where no quantitative data on concentrations were provided on the case sample analysed (129,163,197), or studies where only the range was given but no mean values were reported (142,148,160,167), or only the mean value was reported (131,154,161). One study was excluded (128) as the same data was presented in another publication (138). Studies that were carried out to measure the concentrations after passive exposure to cannabis were also excluded (146,197). Apart from three studies (123,132,137), where hair specimens were collected from post-mortem cases, the hair specimens analysed were from known cannabis users.

#### 5.8.3.1 **THC**

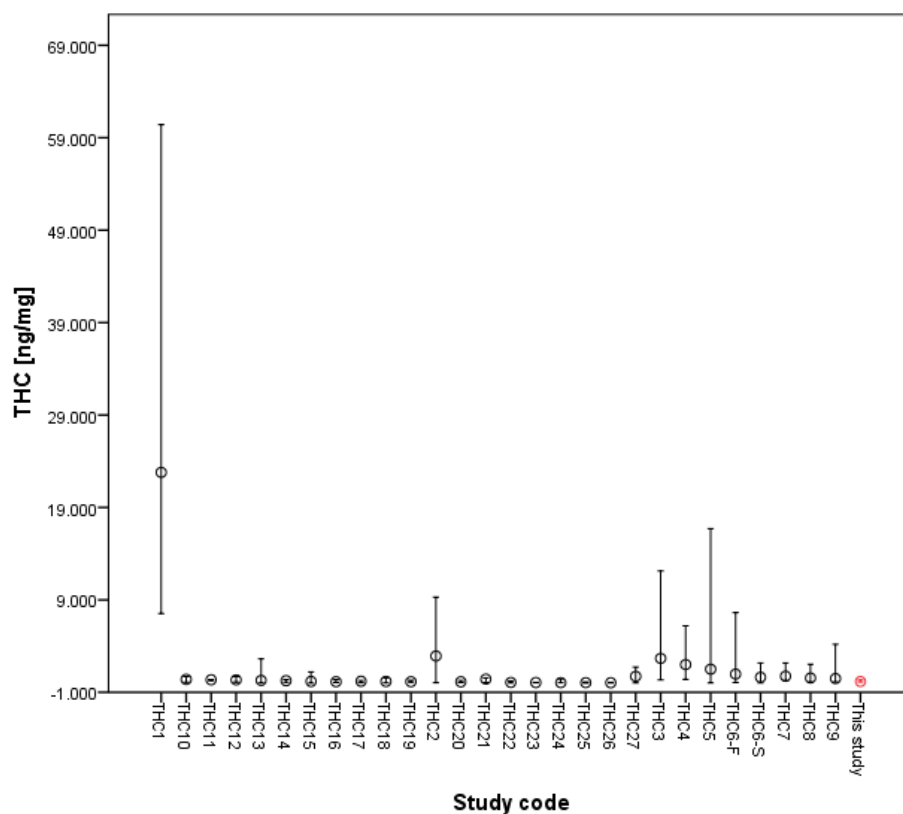
Studies included in the THC concentration comparison are listed in Table 5-10. The mean, minimum and maximum concentrations detected in each study are shown in the same table. To facilitate representation of these data on the high-low diagram, each study was given a code. The concentrations of THC in previous studies were found to range from 0.003 ng/mg to 60.41 ng/mg. Concentrations detected in this study were similar to the wide concentration range of THC detected in other studies. The mean values reported in each study ranged from 0.041 to 22.79 ng/mg and the mean value reported in this study was 0.17 ng/mg. A high-low diagram illustrating the ranges observed for all studies is shown in Figure 5-10. Due to the very high concentration observed in study (THC1), it was necessary to rescale the diagram to allow a proper visual comparison of studies that reported low concentrations as shown in Figure 5-11.



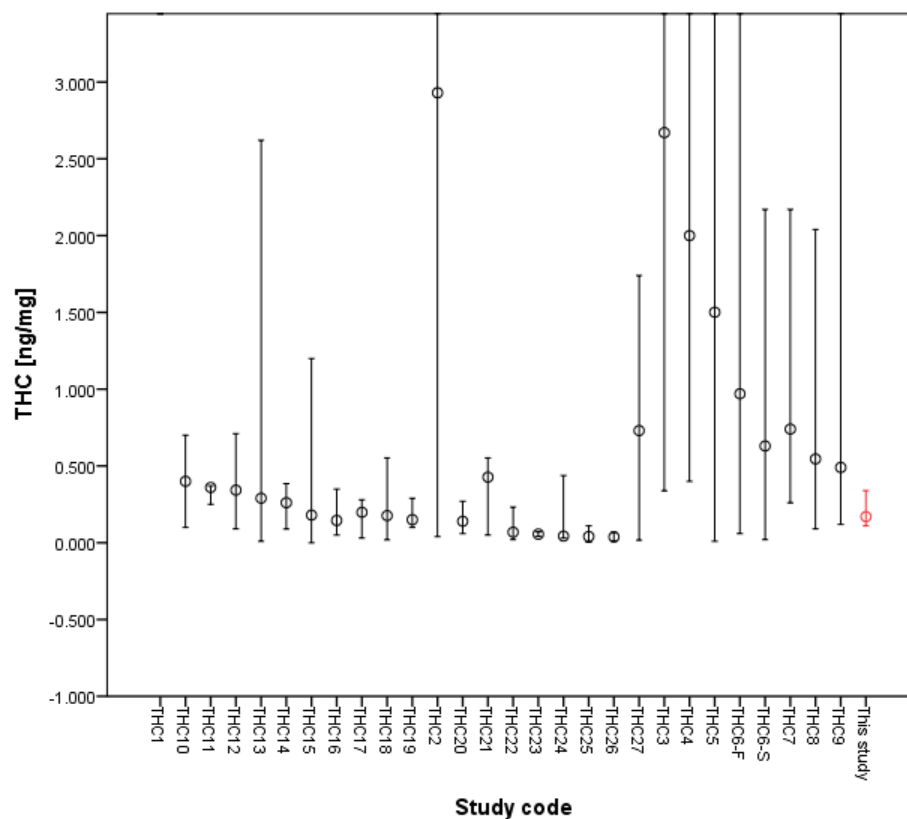
**Table 5-10** List of studies included in comparison of  $\Delta^9$ -tetrahydrocannabinol (THC) concentrations detected in hair matrices. Mean, minimum and maximum concentrations detected in ng/mg are shown. Each study was given a code to use for diagram representation.

Ref.	Author(s) (year)	Code on diagram	Mean	Min.	Max.
(147)	Han <i>et al</i> (2011)	THC1	22.790	7.520	60.410
(130)	Villamor <i>et al</i> (2005)	THC2	2.930	0.040	9.290
(108)	Pichini <i>et al</i> (2015)	THC3	2.670	0.339	12.125
(194)	Moeller and Sachs (1993)	THC4	2.000	0.400	6.200
(191)	Kauret <i>et al</i> (1996)	THC5	1.501	0.009	16.700
(138)	Jurado <i>et al</i> (1996) -SPAIN*	THC6-F	0.970	0.060	7.630
(137)	Cirimele <i>et al</i> (1995)	THC7	0.740	0.260	2.170
(138)	Jurado <i>et al</i> (1996) - FRANCE*	THC6-S	0.630	0.020	2.170
(149)	Nadulski <i>et al</i> (2010)	THC8	0.546	0.090	2.040
(135)	Nadulski & Pragst (2007)	THC9	0.490	0.120	4.200
(124)	Strano-Rossi & Chiarotti (1999)	THC10	0.400	0.100	0.700
(125)	Musshoff <i>et al</i> (2003)	THC11	0.360	0.250	0.370
(145)	Skopp <i>et al</i> (2007)	THC12	0.343	0.090	0.710
(140)	Minoli <i>et al</i> (2012)	THC13	0.290	0.010	2.620
(196)	Koster <i>et al</i> (2014)	THC14	0.261	0.089	0.385
(118)	Moosmann <i>et al</i> (2015)	THC15	0.180	0.000	1.200
(116)	Breidi <i>et al</i> (2012)	THC16	0.145	0.050	0.350
(115)	Baptista <i>et al</i> (2002)	THC17	0.198	0.030	0.280
(150)	Wilkins <i>et al</i> (1995)	THC18	0.177	0.019	0.552
(132)	Cirimele <i>et al</i> (1996)	THC19	0.150	0.100	0.290
(133)	Kim <i>et al</i> (2005)	THC20	0.140	0.060	0.270
(126)	Salomone <i>et al</i> (2012)	THC21	0.427	0.050	0.553
(136)	Emidio <i>et al</i> (2010) - HS	THC22	0.069	0.020	0.232
(144)	Emidio <i>et al</i> (2010) - HF	THC23	0.056	0.041	0.070
(153)	Mieczkowski (1995)	THC24	0.043	0.030	0.438
(134)	Huestis <i>et al</i> (2007)	THC25	0.041	0.003	0.110
(127)	Míguez-Framil <i>et al</i> (2014)	THC26	0.037	0.011	0.068
(159)	Tassoni <i>et al</i> (2015) - group A	THC27	0.730	0.017	1.740

\*The concentrations in this study were reported in two groups, each was representing concentrations detected different country, (F) for France and (S) for Spain.



**Figure 5-10** High-low diagram representing minimum, maximum and mean concentrations of  $\Delta^9$ -tetrahydrocannabinol (THC) detected in each study. The high-low bars and mean marker representing the data in this study are in red.



**Figure 5-11** Zoomed in high-low diagram representing studies with minimum, maximum and mean concentrations of  $\Delta^9$ -tetrahydrocannabinol (THC) detected in each study. The high-low bars and mean marker representing the data in this study are in red.

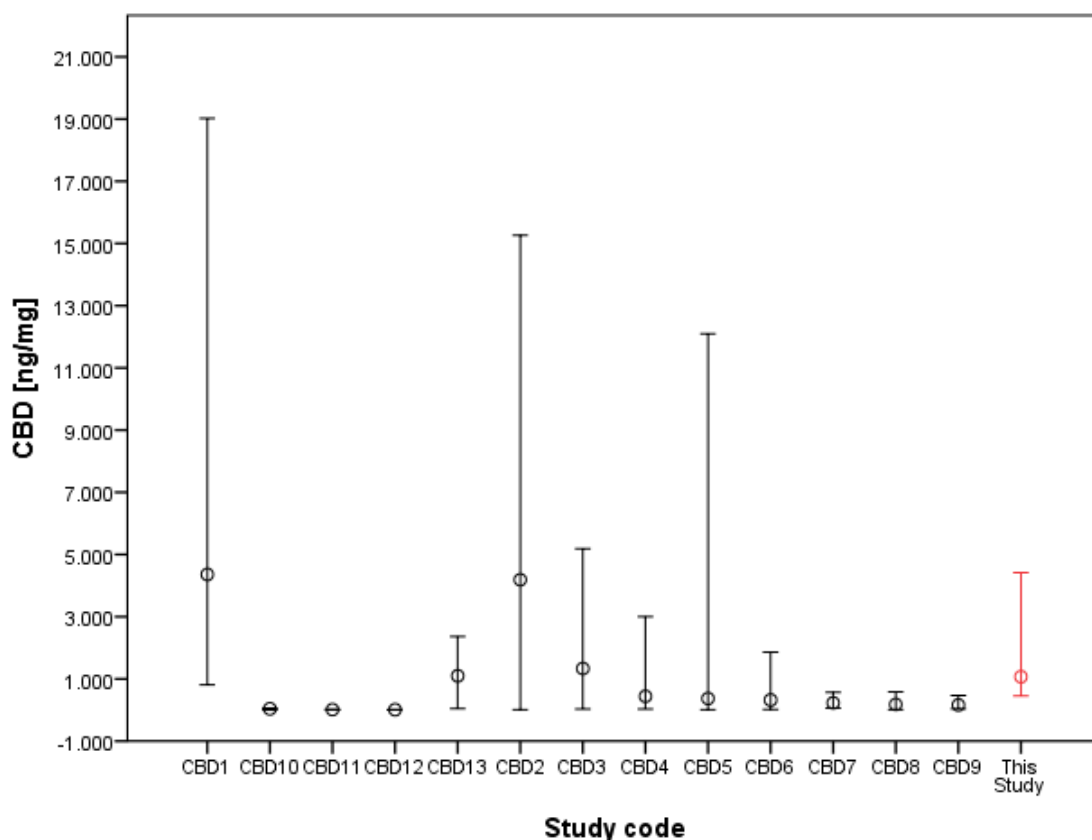
### 5.8.3.2 CBD

Studies included in the CBD concentration comparison are listed in Table 5-11. The mean, minimum and maximum concentrations detected in each study are shown in the same table. To facilitate representation of these data on high-low diagram, each study was given a code. The concentrations of CBD in previous studies were found to range from 0.001 ng/mg to 19.02 ng/mg. The mean values reported across the range of studies ranged from 0.01 to 4.30 ng/mg. The mean value reported in this study was 1.07 ng/mg. A high-low diagram illustrating the ranges observed for all studies is shown in Figure 5-12.

**Table 5-11 List of studies included in comparison of cannabidiol (CBD) concentrations detected in hair matrices. Mean, minimum and maximum detected concentrations in ng/mg are shown. Each study was given a code to use for diagram representation**

Ref.	Author(s) (year)	Code on diagram	Mean	Min.	Max.
(125)	Musshoff <i>et al</i> (2003)	CBD1	4.360	0.810	19.020
(130)	Villamor <i>et al</i> (2005)	CBD2	4.190	0.010	15.260
(149)	Nadulski <i>et al</i> (2010)	CBD3	1.334	0.030	5.190
(132)	Cirimele <i>et al</i> (1996)	CBD4	0.440	0.030	3.000
(135)	Nadulski & Pragst (2007)	CBD5	0.370	0.013	12.100
(126)	Salomone <i>et al</i> (2012)	CBD6	0.323	0.018	1.862
(145)	Skopp <i>et al</i> (2007)	CBD7	0.231	0.060	0.570
(127)	Míguez-Framil <i>et al</i> (2014)	CBD8	0.179	0.008	0.585
(115)	Baptista <i>et al</i> (2002)	CBD9	0.162	0.040	0.470
(133)	Kim <i>et al</i> (2005)	CBD10	0.040	0.020	0.050
(136)	Emidio <i>et al</i> (2010) - HS	CBD11	0.014	0.013	0.020
(144)	Emidio <i>et al</i> (2010) - HF	CBD12	0.010	0.001	0.018
(159)	Tassoni <i>et al</i> (2015) *	CBD13	1.100	0.050	2.360

\*Data from (group A) specimens, nonpreviously treated with acid hydrolysis included.



**Figure 5-12 High-low diagram representing minimum, maximum and mean value for the concentrations of cannabidiol (CBD) detected in each study. The high-low bars and mean marker representing the data in this study are in red.**

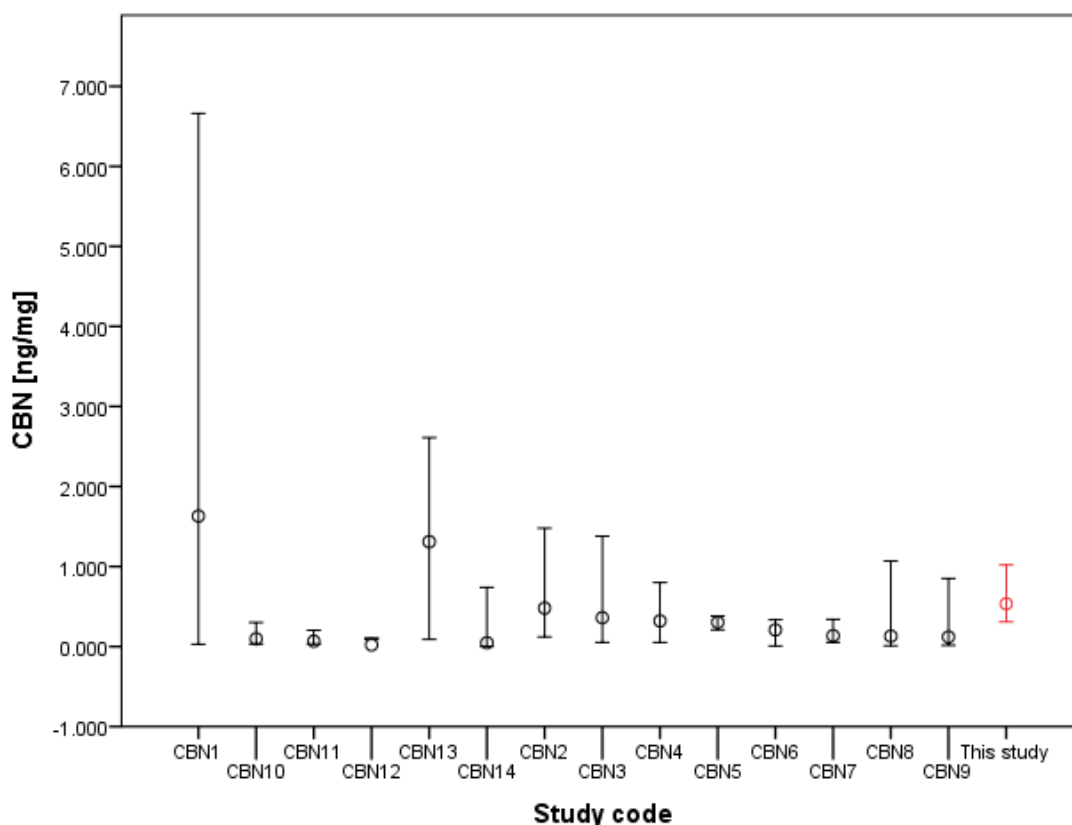
### 5.8.3.3 CBN

Studies included in the CBN concentration comparison are listed in Table 5-12. The mean, minimum and maximum concentrations detected in each study are shown in the same table. To facilitate representation of these data on the high-low diagram, each study was given a code. The concentrations of CBN in previous studies were found to range from 0.008 ng/mg to 6.66 ng/mg. The mean values reported across the range of studies ranged from 0.021 to 1.63 ng/mg. The mean value reported in this study was 0.53 ng/mg. A high-low diagram illustrating the ranges observed for all studies is shown in Figure 5-13.

**Table 5-12 List of studies included in comparison of cannabinol (CBN) concentrations detected in hair matrices. Mean, minimum and maximum detected concentrations in ng/mg are shown. Each study was given a code to use for diagram representation**

Ref.	Author(s) (year)	Code on diagram	Mean	Min.	Max.
(130)	Villamor <i>et al</i> (2005)	CBN1	1.630	0.030	6.660
(125)	Musshoff <i>et al</i> (2003)	CBN2	0.480	0.120	1.480
(133)	Kim <i>et al</i> (2005)	CBN3	0.360	0.050	1.380
(149)	Nadulski <i>et al</i> (2010)	CBN4	0.320	0.050	0.800
(115)	Baptista <i>et al</i> (2002)	CBN5	0.303	0.210	0.380
(127)	Míguez-Framil <i>et al</i> (2014)	CBN6	0.209	0.008	0.335
(145)	Skopp <i>et al</i> (2007)	CBN7	0.136	0.050	0.340
(132)	Cirimele <i>et al</i> (1996)	CBN8	0.130	0.010	1.070
(135)	Nadulski & Pragst (2007)	CBN9	0.120	0.016	0.850
(136)	Emidio <i>et al</i> (2010) - HS	CBN10	0.096	0.031	0.300
(126)	Salomone <i>et al</i> (2012)	CBN11	0.066	0.031	0.205
(144)	Emidio <i>et al</i> (2010) - HF	CBN12	0.021	0.090	0.107
(159)	Tassoni <i>et al</i> (2015) *	CBN13	1.310	0.090	2.610
(118)	Moosmann <i>et al</i> (2015)	CBN14	0.048	0.000	0.740

\*Data from (group A) specimens, nonpreviously treated with acid hydrolysis included.



**Figure 5-13 High-low diagram representing minimum, maximum and mean value for the concentrations of cannabiniol (CBN) detected in each study. The high-low bars and mean marker representing the data in this study are in red.**

#### 5.8.3.4 THC-COOH

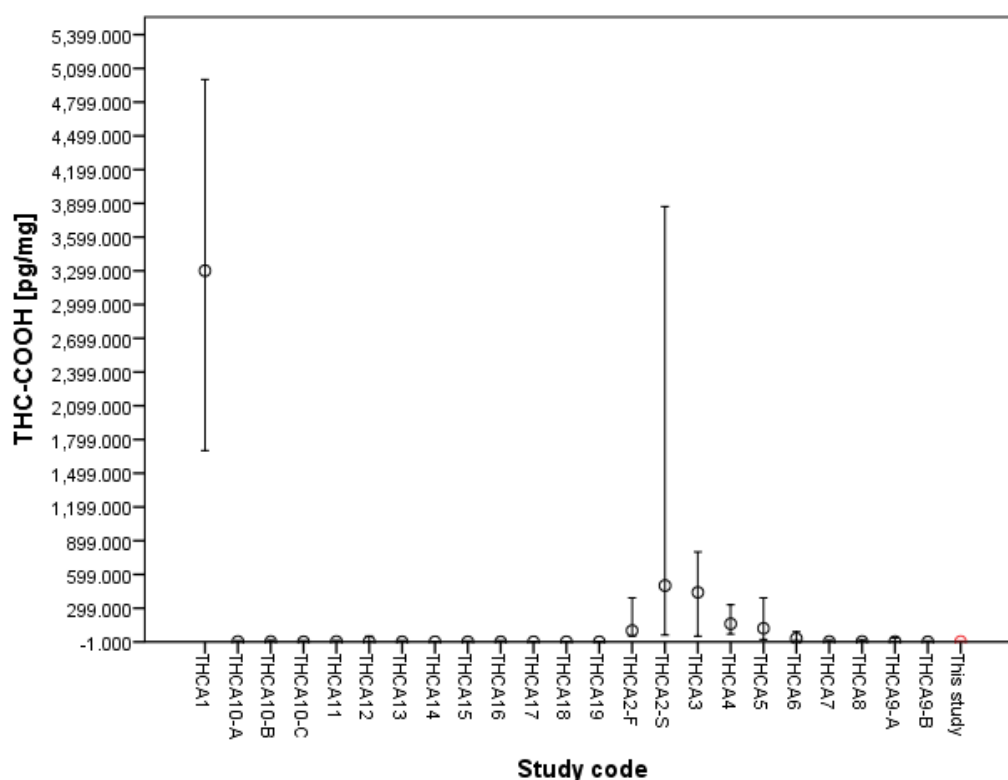
Studies included in the THC-COOH concentration comparison are listed in Table 5-13. The mean, minimum and maximum concentrations detected in each study are shown in the same table. To facilitate representation of these data on high-low diagram, each study was given a code. The concentrations of THC-COOH in previous studies were found to range from 0.02 pg/mg to 5000 pg/mg. The mean values reported across the studies ranged from 0.259 to 3300 pg/mg. The mean value reported in this study (sample B) was 2.58 pg/mg. A high-low diagram illustrating the ranges observed for all studies is shown in Figure 5-14. Due to the very high concentration observed in studies THCA1 to THCA6, it was necessary to rescale the diagram to allow a proper visual comparison of studies that reported low concentrations as shown in Figure 5-15.

**Table 5-13** List of studies included in comparison of 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) concentrations detected in hair matrices. Mean, minimum and maximum detected concentrations in pg/mg are shown. Each study was given a code to use for diagram representation

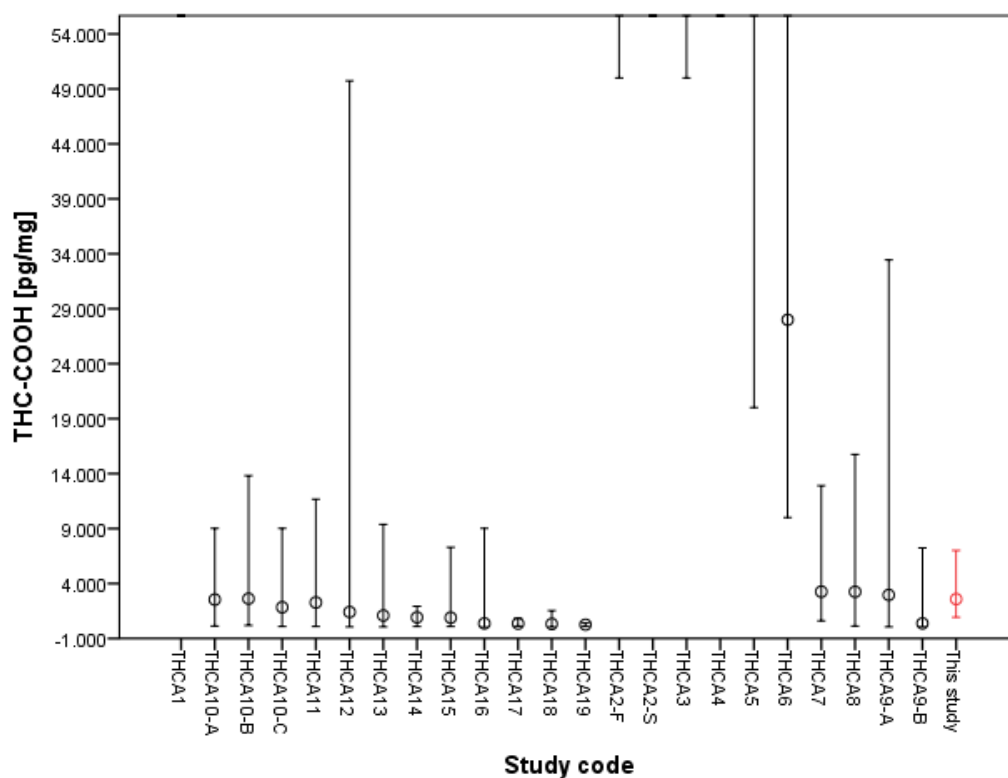
Ref.	Author(s) (year)	Code on diagram	Mean	Min.	Max.
(194)	Moeller and Sachs (1993)	THCA1	3300	1700	5000
(138)	Jurado <i>et al</i> (1996)-SPAIN	THCA2-S	500	60	3870
(130)	Villamor <i>et al</i> (2005)	THCA3	440	50	800
(137)	Cirimele <i>et al</i> (1995)	THCA4	160	70	330
(123)	Kintz <i>et al</i> (1995)	THCA5	120	20	390
(138)	Jurado <i>et al</i> (1996)-France	THCA2-F	100	50	390
(115)	Baptista <i>et al</i> (2002)	THCA6	28	10	90
(152)	Moore and Guzaldo (2001)	THCA7	3.26	0.6	12.9
(162)	Park <i>et al</i> (2014)	THCA8	3.25	0.13	15.75
(155)	Han <i>et al</i> (2011) (A)*	THCA9-A	2.96	0.06	33.44
(143)	Han <i>et al</i> (2011) (B)**	THCA10-B	2.62	0.19	13.82
(143)	Han <i>et al</i> (2011) (A)**	THCA10-A	2.54	0.14	9.01
(147)	Han <i>et al</i> (2011)	THCA11	2.27	0.1	11.68
(143)	Han <i>et al</i> (2011) (C)**	THCA10-C	1.84	0.1	9.01
(140)	Minoli <i>et al</i> (2012)	THCA12	1.41	0.05	49.74
(156)	Kim <i>et al</i> (2011)	THCA13	1.09	0.05	9.38
(141)	Moore <i>et al</i> (2006)	THCA14	0.922	0.09	1.94
(134)	Huestis <i>et al</i> (2007)	THCA15	0.89	0.1	7.3
(155)	Han <i>et al</i> (2011) (B)*	THCA9-B	0.37	0.05	7.24
(158)	Jones <i>et al</i> (2013)	THCA16	0.36429	0.02	9.011
(139)	Kim and In (2007)	THCA17	0.35	0.14	0.85
(153)	Mieczkowski (1995)	THCA18	0.322	0.03	1.53
(108)	Pichini <i>et al</i> (2015)	THCA19	0.259	0.09	0.39

\*THCA9 A and B are two sets of data from the same publication.

\*\*THCA10 A, B and C are two sets of data from the same publication.



**Figure 5-14 High-low diagram representing minimum, maximum and mean value for the concentrations of 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) detected in each study. The high-low bar and mean dot representing the data in this study are in red.**



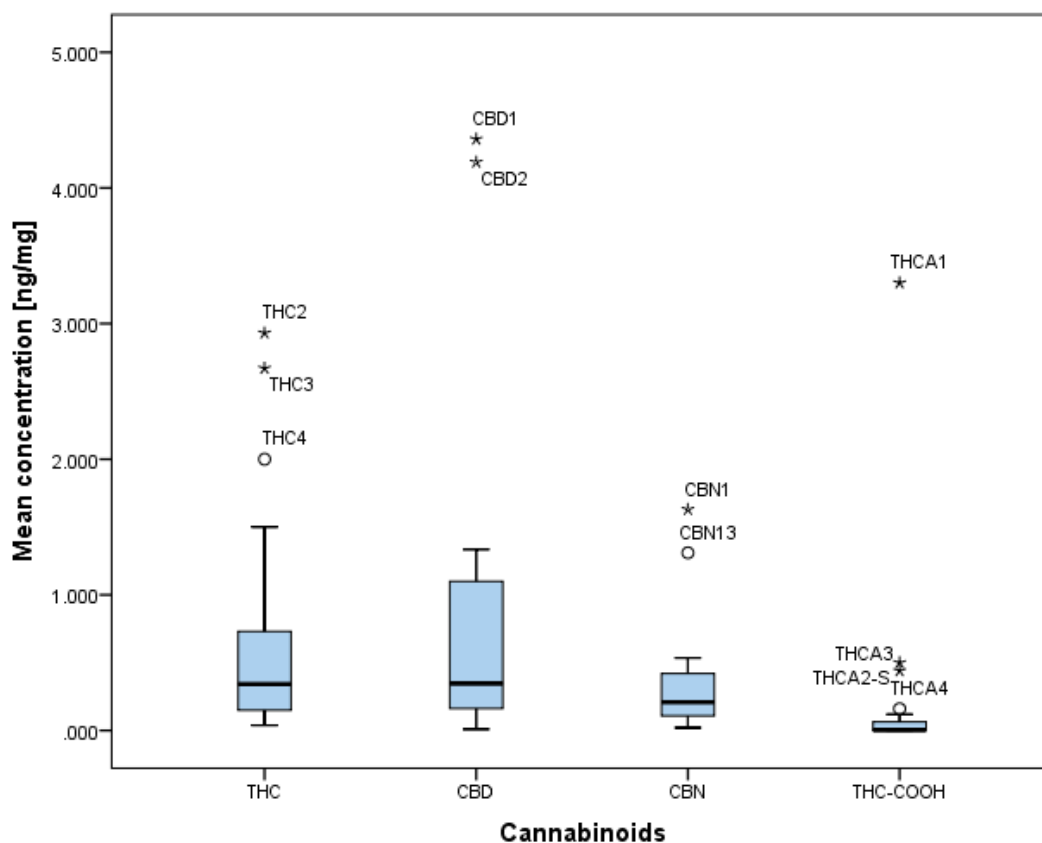
**Figure 5-15 Zoomed-in high-low diagram representing minimum, maximum and mean value for the concentrations of 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid (THC-COOH) detected in each study. The high-low bar and mean marker representing the data in this study are in red**



#### 5.8.3.5 Conclusions from comparison with other studies

Despite the fact that the reported concentrations for THC in hair in the literature were found to vary over a wide range, the mean concentrations of THC, CBN and CBD in the studies included in this comparison show insignificant difference when tested using the Kruskal-Wallis test. Figure 5-16 shows a boxplot diagram of the mean concentrations of THC, CBD, CBN and THC-COOH in all studies included for comparison in addition to this study. It is possible that this greater variation in THC concentrations is a result of using inappropriate derivatisation reagent in some studies. The use of perfluorinated anhydrides alone or coupled with perfluoroalcohols, such as HFIP/TFAA or PFPOH/PFPA, will results in identical retention times and mass spectra for THC and CBD. This may explain the elevated levels of THC reported in some studies that were included in the comparison (137,138,147,191).

The current LOQ of 4 ng total (0.08 ng/mg based on 50 mg hair) for THC in this study was close to the SOHT recommended cut-off value (0.1 ng/mg). However, there were only four hair specimens (from three users out of twenty) that had quantifiable THC peaks above 4 ng total. Based on this and the concentrations reported in the other studies, a lower cut-off might be more appropriate. There is no recommended cut-off for CBD and CBN. However, based on the obtained concentrations in this study and comparison of the mean concentrations reported in the literature, a similar cut-off to THC may be appropriate.



**Figure 5-16** Boxplot diagrams representing the median and interquartile range of mean concentrations (ng/mg) of  $\Delta^9$ -tetrahydrocannabinol (THC) (n=29), cannabidiol (CBD)(n=14), cannabinol (CBN) (n=15) in hair of cannabis users.

Kruskal-Wallis test was carried out to compare the mean concentrations of THC, CBD and CBN. There was no significant difference ( $p>0.05$ ) in the distribution of THC, CBD and CBN concentrations. The mean concentration from THC1 is not included in the statistical analysis nor represented in the boxplot.

The current LOQ for THC-COOH of 50 pg total (1 pg/mg based on 50 mg hair) was capable of detecting 74.3% and 66.7% of non-daily and daily cannabis users, respectively. It would be necessary to improve detection sensitivity in order to detect lower concentrations. Improvement of the detection sensitivity for THC-COOH would require further work to improve the clean-up of the structurally similar lipophilic organic acids resulting from the hair matrix which elevate the background noise (163). Amongst the studies that have been reported, there has been a wide range of concentrations reported for THC-COOH. This broad range may be attributed to the variance in instrumental capabilities and analytical method sensitivity. The mean values across all the studies ranged from 0.26 to 3300 pg/mg ( $m=194.8$  pg/mg). There were 6 studies which detected much higher concentrations than the other 17 studies (115,123,130,137,138,194). When these studies were excluded, the means ranged from 0.25 to 3.26 pg/mg (1.6 pg/mg).

The mean concentration detected in this study was 2.3 pg/mg which is similar to the values reported in previous literature.

#### **5.8.4 Correlation of concentrations detected with self-reported cannabis use**

Twenty specimens from known cannabis users were analysed in this study. It was found that both analytical methods are suitable for detecting THC, CBD, CBN and THC-COOH in both types of users. However, there was no difference in the concentrations of THC, CBD, CBN and THC-COOH detected for weekly and daily users. As non-daily and daily use is not reflective of the actual dose consumed by a user, the weekly use score was calculated based on information provided by users on their use history. This score is an estimation of number of joints/spliffs they had smoked on a weekly basis in the three months prior to admission to the addiction hospital. Users reported a constant behaviour of smoking over the three months, therefore, the correlation between the concentrations in the hair segment that represent the last three months and weekly use score was investigated. Statistical analysis showed no correlation between the concentrations of the individual analytes detected and weekly use score. The lack of correlation can be explained by the following factors including: variability of hair-growth cycle, multiple mechanisms of drug incorporation and considerable variation in uptake of drug from blood to hair, influence of cosmetic treatments and hygienic practices, uncertainty of dosages ingested by abusers (number of joints/spliffs and days), typical underestimation of self-reported doses, unknown purity of compounds (THC and CBD content), rate of sweating and amount of apocrine and sebaceous gland secretions between individuals and degree of exposure to cannabis smoke. Better correlation may have been achieved if these factors were taken into account with a greater sample size.

### **5.9 Conclusion**

This chapter has demonstrated that the two methods developed and validated in chapters 3 and 4 are suitable for the analysis of authentic hair specimens for the detection of THC, CBD, CBN and THC-COOH. The authentic hair specimens were collected from cannabis users, who were admitted to Al-amal addiction hospital, Jeddah, Saudi Arabia. Users reported the number of joints/spliffs they smoked in

a day and the number of days in a week. The first method on the standard GC-MS was found to be suitable to prove exposure by detection of the main cannabinoids, THC, CBD and CBN. While the other method using the 2D GC-MS was found suitable to prove ingestion by detection of THC-COOH. Similar to conventional biological samples, the detection of other cannabinoids (cannabinol (CBN), cannabidiol (CBD)) in hair serves as a plausible control due to their higher stability as compared to THC. There was no correlation between the concentrations of all analytes and the frequency of use or self-reported doses. A lower LOQ for the main cannabinoid THC may increase the detectability. CBD and THC-COOH had the highest detection rate. 11-OH-THC was not detected in any case sample. This was expected due to the higher LOQ. The use of fraction 'B' of sample (A) to carry out further clean-up using SPE for THC-COOH was proven to be an acceptable approach. However, case samples with much lower concentrations may become undetectable.

## Chapter 6 Introduction to Environmental Tobacco Smoke (ETS) Exposure

### 6.1 Introduction

#### 6.1.1 Indoor air pollutants

According to the United States Environmental Protection Agency (EPA), air pollution happens when the air contains gases, dust, fumes or odour in harmful amounts. That is, amounts which could be harmful to human and animal health or comfort or which could cause damage to plants and materials. In the World Health Organisation (WHO) 2014 report, it was estimated that air pollution, household and ambient, has caused the deaths of around 7 million people worldwide in 2012 which is about one in eight of total global deaths (199). This finding more than doubles previous estimates and confirms that air pollution is now the world's largest single environmental health risk. From the same report, 4.3 million deaths, globally, were attributable to indoor (household) air pollution alone. The main sources of indoor air pollution worldwide include indoor combustion of solid fuels, outdoor air pollutants, emissions from construction materials and furnishings, tobacco smoking, and inadequate maintenance of ventilation and air conditioning systems. While some indoor air pollutants, such as environmental tobacco smoke (ETS), are gaining increasing attention globally, profiles of indoor air pollutants and the consequential health risks are generally very different in developed and developing countries.

In Europe, ETS has been of concern since 1987 when the first edition of the air quality guidelines for Europe included a chapter on radon and an annex on tobacco smoke, indoor air pollutants with substantial adverse public health impacts. In the second edition published in 2000, a section on indoor air pollutants and added manmade vitreous fibres to radon and tobacco smoke was provided (200). Since 1 July 2007, smoke-free workplace laws have been in effect across the whole of the UK.

In the United States of America (USA), tobacco smoke has been identified as a toxic air contaminant since 2005 (201). The Surgeons General and EPA have undertaken a more proactive role in informing the American public on health risks

associated with environmental tobacco smoke exposure (202-208). As smoking bans in the United States are entirely a product of state and local criminal and occupational safety and health laws, there is no national-wide ban of smoking so far. In 2000, California became the first state to ban smoking in bars and restaurants. As of July, 2016, 25 States have enacted Statewide bans on smoking in workplaces and restaurants and bars, according to the American Nonsmokers Right Foundation (ANRF) (209).

In Singapore, the Health Promotion Board (HPB) estimated that ETS caused twice as many deaths as all other types of air pollution combined (210). The 2005 WHO global update of the air quality guidelines drew attention to the large impact on health of indoor air pollution in developing countries (211).

## 6.2 Environmental Tobacco Smoke (ETS) Exposure

Smoking is a widely acknowledged threat to human health. Environmental tobacco smoke (ETS), also known as second-hand smoke (SHS), involuntary smoking (IS) or passive smoking (PS), is a significant contaminant of indoor air following active smoking. Although ETS is not a leading human cause of air pollution, it poses a substantial health risk especially after long-term exposure in enclosed spaces. It is the main source of exposure to a large number of chemicals that are known to be hazardous to human health. In a study conducted in Italy in 2004, the particulate matter released from three lit cigarettes was found to be ten times higher than the output from a diesel engine after 30 minutes in a controlled environment (212). Tobacco can be smoked in different forms such as smoking of a cigarette, cigars, pipes, and water pipes<sup>2</sup> such as shisha, hookah, narghile, or hubble-bubble. However, cigarettes and bidis<sup>3</sup> were found to be the main sources of exposure among non-smokers or smokers from secondary smoking by others. ETS contains approximately 50 chemicals recognized as known and/or possible human carcinogens, other animal carcinogens, and many toxic and irritant agents (213). Since 1928, there have been reports published drawing attention toward

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<sup>2</sup> Water pipes are known by other various names such as gaza, hookah, narghile, shisha, hubble-bubble and, are commonly smoked in the Eastern Mediterranean region, in some parts of Asia including India, and in North Africa. A typical modern water pipe consists of a head with holes in the bottom, a metal body, a glass water bowl, and a flexible hose with a mouthpiece

<sup>3</sup> Bidis are small, thin, hand-rolled cigarettes primarily made of coarse and uncured tobacco. These kind of cigarettes account for about 60% of smoked tobacco products in India.

the harmful effects of ETS (214). Evidence relating to the negative health effects of ETS have accumulated over the years from many studies carried out in different parts of the world, however, ETS remains a common indoor air pollutant across the world. Approximately 85-90% of the smoke from each cigarette turns out in the air to be ETS.

Two types of smoke are produced by a lit cigarette; mainstream and sidestream smoke. Mainstream smoke (MS) is the smoke that is inhaled by the smoker through the filter tip of the cigarette, and then exhaled. While sidestream smoke (SS) from the burning end of the cigarette goes straight into the atmosphere. Toxic chemicals from ETS pollution are detected well beyond the period of active smoking, and contaminate furniture, clothes, food, equipment and other materials. Even in well-ventilated places, these toxins can persist for weeks and months after smoking (215).

### **6.2.1 Composition of tobacco smoke**

ETS usually consists of approximately 85% sidestream smoke and 15% mainstream smoke. Sidestream smoke, which has a higher temperature and does not pass through the cigarette's filter tip, has higher concentrations of chemicals than mainstream smoke. Analysis of mainstream and sidestream cigarette smoke particulate matter by laser desorption mass spectrometry has revealed that they have a very similar composition (216). However, other toxic compounds and combustion products may vary in their concentrations (217). Tobacco smoke is a mixture of more than 5300 compounds, 500 of which have been measured in mainstream and sidestream smoke (218). Of these by-products of ETS, 172 are known toxic substances. There are over 70 carcinogens in tobacco smoke that have been evaluated by the International Agency for Research on Cancer (IARC) monographs programme as having adequate evidence for carcinogenicity in either laboratory animals or humans (219). Many tobacco alkaloids are present in the ETS, including nornicotine, anatabine and anabasine, however, the addictive properties of tobacco smoke are believed to be due to the presence of nicotine, the principal tobacco alkaloid in smoke (220).

### 6.2.2 Implementation and effectiveness of smoke-free policies

As tobacco smoke is classified as carcinogenic, the impact of environmental health on humans becomes more obvious. In fact, this has encouraged policymakers, public health strategists and air quality experts to prioritise clean air. Since the WHO encouraged participant nations to follow Article 8 of the Framework Convention on Tobacco Control<sup>4</sup> (FCTC) (221), smoke-free policies have come into effect worldwide to protect people from ETS. Following the FCTC treaty, the WHO published in 2007 guidelines on the protection from tobacco smoke, to support countries' efforts for implementation of Article 8 (222). Since then, the WHO published a series of reports that follow the status of the tobacco epidemic and the effectiveness of policies implemented to stop it. The first WHO report on the global tobacco epidemic was published in 2008. This report provided countries with a guideline to reverse the highly destructive global tobacco epidemic and introduced the so called 'MPOWER<sup>5</sup>' package (223). The second report was published in 2009, this report concentrated on the progress made in implementing the smoke-free policies and reported a worldwide implementation in enclosed workplace and public places, and public transportation (224). Ireland was the first country with comprehensive smoke-free legislation implemented in 2004 before FCTC came into force in 2005, followed by countries like Norway, New Zealand, Italy, Spain, Uruguay, England and many provinces or states in Canada, the USA and Australia. In Scotland, legislation to ban smoking in most enclosed public places was introduced in March 2006. Following the expansion in implementing the smoke-free policies, many studies have been conducted to measure the impact of the new legislation on public health and smoking cessation in different countries (225-230). The third WHO report, published in 2011, examined in depth the two principal strategies to provide health warnings - labels on tobacco product packaging and anti-tobacco mass media campaigns. It provides a comprehensive overview of the evidence base for warning people about the harms of tobacco use as well as country-specific information on the status of these measures.

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<sup>4</sup> FCTC is a treaty adopted by the 56th World Health Assembly on 21 May 2003.

<sup>5</sup> MPOWER encompasses six policies proposed to counter the tobacco epidemic and reduce its deadly toll.



The fourth report provided a special focus on legislation to ban tobacco advertising, promotion and sponsorship (TAPS) in WHO Member States and an in-depth analyses of TAPS bans were performed, allowing for a more detailed understanding of progress and future challenges in this area. The latest report, published in 2015, has a particular focus on tobacco taxation and includes in-depth analyses of tobacco taxes and prices in all WHO Member States, allowing a detailed understanding of progress and future challenges in this area. The WHO 2015 report estimated that about 2.8 billion people (40% of the world's population) in more than half of the world's countries, have implemented at least one MPOWER measure at the highest level of achievement. Despite the wide enforcement of smoke-free legislation worldwide, some sources reported that around 93% of the world's population is still living in countries not covered by fully smoke-free public health regulations (214). Moreover, some studies showed that smoking ban policies in public places have led to an increase in indoor exposure, particularly in young children, to tobacco smoke as smokers shifted smoking from public to private places (231).

### **6.3 Measurement of ETS Exposure**

It is difficult to measure the exposure of a passive smoker to environmental tobacco smoke. Many factors have a role to play in determining the degree of exposure which in turn makes it difficult to standardise the exposure levels. These include, the number and type of cigarettes, the number of smokers present in the room, the rate and manner of smoking, the room size, temperature, air exchange rate (ventilation) and humidity. All of these factors have to be considered carefully when interpreting the data regarding constituents of ETS obtained from indoor space.

Historically, ETS can be assessed by different means such as questionnaires, air monitoring, modeling of concentrations, or biological markers. In the 1980's it was established that cigarette smoking is a powerful source of fine indoor airborne particulate matter  $<2.5 \mu\text{m}$  ( $\text{PM}_{2.5}$ ) and gas phase nicotine which was found to be a sensitive and specific marker of ETS. Unlike nicotine, many other markers, although present in the ETS, are not specific to tobacco smoke, such as  $\text{PM}_{2.5}$ , may arise from a range of sources. So far, none of the markers in use, however, meet all of the 1986 National Research Council (NRC) criteria. Additionally, there is no

single constituent that will reveal the full disease risk from the complex mixture that comprises ETS. It can be concluded that the purpose of the study dictates the choice of method for measuring ETS.

According to the 1986 NRC report, the ideal marker for ETS exposure measurement should be; 1) exclusive (or almost exclusive) to tobacco smoke, 2) a component of tobacco smoke that is present in an adequate amount so that it can be measured even at low ETS levels, and 3) present at a fairly constant ratio across different brands of cigarettes to other tobacco smoke constituents (or contaminants) of interest. In addition to the above criteria, it is important to obtain reliable information on the amount, transport, and the fate of such chemicals in normal indoor environments. The majority of ETS exposure studies have used either  $PM_{2.5}$  as an indicator to the degree of exposure due to its extensive release from the tobacco combustion within indoor spaces or nicotine due to its exclusive presence in tobacco smoke. Other proxy constituents have been measured in a number of studies as indicators of ETS exposure either in personal or indoor space monitoring. Carbon monoxide, cotinine, nitrogen oxides, acrolein, nitroso-compounds, and benzo[a] pyrene are some of the compounds or classes of air contaminants that have been measured under field conditions as indicators of ETS exposure. Biomarkers specific to ETS can be targeted in different biological samples to indicate the level of exposure.

### 6.3.1 Biological markers

Nicotine and its metabolites, and metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are considered to be specific biomarkers to ETS exposure. Metabolites of nicotine (cotinine, trans-3'-hydroxycotinine and their glucuronides, and nicotine glucuronide) and NNK (NNAL (4- [methylnitrosamino]-1-[3-pyridyl]-1-butanol) and its glucuronides) can be measured in people exposed to ETS, with high sensitivity in different biological matrices. The selection of an ETS biomarker and biological matrix is based on many factors including the aim of the study, the scientific or public health knowledge gap, subjects, and available resource. A combination of different biomarkers, or matrices, is often the best approach (232).

About 75% of the nicotine absorbed by the body is metabolised to form cotinine (220). Cotinine concentrations are more constant during the day with a longer half-life ( $t_{1/2}$ ) (about 16 hours) than nicotine (about 2 hours). This makes cotinine the preferred biomarker to measure ETS exposure in blood, saliva and urine. Despite the fact that cotinine has a similar half-life ( $t_{1/2}$ ) in blood, saliva and urine, the cotinine concentrations in urine averaged four to six times higher than those in blood or saliva, making urine a more sensitive and convenient matrix to test for low-level exposure (233). Up to 15 metabolites of nicotine including nicotine N-oxide, cotinine, norcotinine, cotinine N-oxide and trans 3-hydroxy cotinine have been detected in urine [2]. Many studies reported the use of urine tests to measure the amount of cotinine in the body (234-236). Cotinine use as a biomarker of environmental tobacco smoke exposure was reviewed by Benowitz (237).

Unlike biological fluids, deposited nicotine on the outer surface of hair can be targeted to measure the ETS exposure. This is because it does not undergo a further enzymatic biotransformation. Chemical transformation of nicotine is possible either before adhering to the hair during burning of tobacco or later by atmospheric oxidation and/or reacting with hair treatment products (238). However, it is assumed that the slow reaction speed at ambient temperature will not degrade a high percentage of nicotine, and therefore, nicotine is still a valid ETS exposure indicator. Al-delaimy *et al* measured and compared the two most popular biomarkers, hair nicotine and urine cotinine with questionnaire reports of ETS exposure for 322 children aged 3-27 months and concluded that hair nicotine is a more accurate biomarker, especially for assessment of long-term ETS exposure (239). In a recently published study, Kim *et al* conducted a similar experiment but this time, comparing utility of hair nicotine and salivary cotinine, as a biomarker of long-term ETS exposure, with questionnaire reports of ETS exposure for 289 adult participants for smokers (N = 109) and non-smokers with exposure history (N = 105) and non-smokers with no exposure history (N = 77). According to the authors, hair nicotine was found to be a better indicator for long-term exposure, while salivary cotinine levels showed a better correlation with recent tobacco exposure. Both hair nicotine and its main metabolite cotinine have been most frequently measured in different published studies for different purposes (240-243).

## 6.4 Health Effects of ETS

ETS exposure is one of the most common avoidable health threats in the community. Worldwide, it is predicted that approximately one third of adults are regularly exposed to ETS. On the European list of the ten most common causes of severe human illness, smoking is ranked second. According to the 2009 WHO report, the estimated number of deaths that are attributable to tobacco is about 5 million people each year worldwide, of which 28% were expected to be children (224). The EPA has classified tobacco smoke pollution as a known cause of cancer in humans (Group A carcinogen). It estimates that ETS causes approximately 3,000 lung cancer deaths and 37,000 heart disease deaths in non-smokers each year. Tobacco-specific nitrosamines (TSNAs) comprise one of the most important groups of carcinogens in tobacco products. These nitrosamine carcinogens are formed by the action of nitrous acid on nicotine, nor nicotine, anabasine, and anatabine during curing and processing of tobacco (220). Eight tobacco-specific nitrosamines have been identified. N-Nitrosornicotine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) are the most carcinogenic. The higher cardiovascular disease risk amongst smoke-exposed non-smokers is attributed to the oxidant gas exposure from ETS as it is believed to cause inflammation and subsequent endothelial dysfunction (244). Up to the present time, research has been focused on the health implications to children and adult non-smokers. Studies have established that ETS exposure among populations of different ages is a general health risk and exerts serious consequences on the cardio-respiratory system (245,246). Of all deaths attributable to second-hand tobacco smoke, 31% occur among children and 64% occur among women.

### 6.4.1 Health effects of ETS on neonates

In pregnant women, the risk includes reduced foetal growth, low birth weight, pre-term delivery and sudden infant death (201,247). The other risks are spontaneous abortion, intrauterine growth retardation, adverse impacts on cognition and behaviour, allergic sensitization, elevated decreased pulmonary function growth and adverse effects on fertility or fecundity, and elevated risk of stroke (201).

### 6.4.2 Health risks to children

Tobacco smoke pollution is especially harmful to young children. Worldwide, at least 40% of children are regularly exposed to ETS after birth (214). EPA estimates that ETS is responsible for between 150,000 and 300,000 lower respiratory tract infections in infants and children under 18 months of age annually, resulting in between 7,500 and 15,000 hospitalizations each year (200). Tobacco smoke pollution is harmful to children with asthma. The EPA estimates that for between 200,000 and one million asthmatic children, exposure to ETS worsens their condition (248). Although ETS exposure is a well-recognised risk factor for cancer among adults, there was evolving evidence that it might also be linked with childhood cancers (247,249,250). Diseases such as adult cardiovascular disease are now believed to be progressive inflammatory diseases started in childhood (251).

### 6.4.3 Health effects of ETS on animals

There are only a few studies in the literature that have investigated the effects of smoking on animals. Tobacco smoke contains three known animal carcinogens (248). The N-nitrosodimethylamine (NDMA), which is an animal carcinogen of a volatile nature, reported to be present in tobacco smoke and released 20-100 times higher in sidestream (SS) than in main stream (MS) smoke (248). The association between ETS and disease has been difficult to prove as assessed by questionnaire-based studies (252-254). However, in dogs, ETS exposure appears to increase the relative risk of cancer of the lung (255), nasal cavity and paranasal sinuses(254). Hawkins *et al* investigated the relationship between ETS exposure and chronic coughing in dogs but no association was confirmed (256). In contrast, Roza and Viegas reported an association of ETS exposure with elevated numbers of macrophages and lymphocytes and macrophage anthracosis in bronchioalveolar lavage fluid collected from Yorkshire terriers (257). In pet cats, the finding of research carried out by Bertone *et al* suggested that the passive exposure to tobacco smoke may increase risk of malignant lymphoma and oral squamous cell carcinoma (258,259).

## 6.5 Nicotine and Related Alkaloids in Tobacco Products

The basic raw materials used in the tobacco industry are plants of the genus *Nicotiana*, belonging to the Solanaceae family (Figure 6-1). For which there are 67 sub-species originating from the Americas and Australia. The most common species used in the industry is *Nicotiana tabacum* L. At present, these plants are cultivated in many other parts of the world for production of tobacco leaf for cigarettes.



Figure 6-1 *Nicotiana tabacum* L

Nicotine is the major tobacco alkaloid occurring at about 1.5% by weight in commercial cigarette tobacco and comprising about 95% of the total alkaloid content. The minor alkaloids nornicotine, anatabine and anabasine are the most abundant in most tobacco products. However, nornicotine levels are highest in cigar tobacco, anatabine levels are lowest in chewing tobacco and oral snuff, and anabasine levels are lowest in chewing tobacco (260). In general, the tobacco alkaloids are not considered as carcinogenic. Figure 6-2 shows the chemical structure of most abundant tobacco alkaloids.

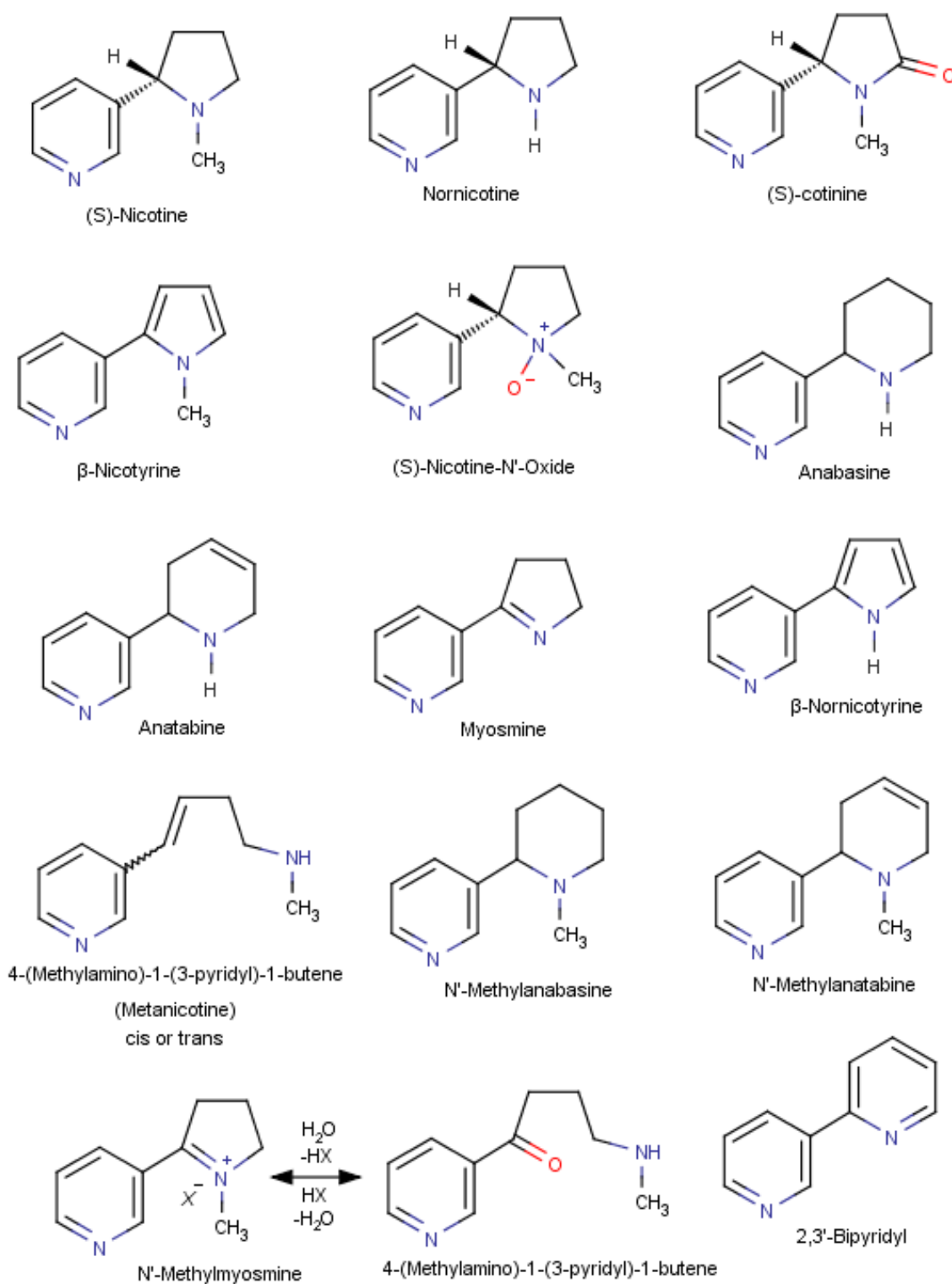


Figure 6-2 Structures of tobacco alkaloids.

Nicotine, which was first isolated in 1828 from tobacco, is an extremely toxic alkaloid that leads to stimulation of autonomic ganglia and the central nervous system (CNS) (261).

### 6.5.1 Chemical properties of Nicotine

Nicotine is also called 3-(1-methyl-2-pyrrolidinyl) pyridine according to the IUPAC<sup>6</sup> nomenclature. As illustrated in Figure 6-3, it is a bicyclic compound with both a pyridine and a pyrrolidine ring. The molecule possesses an asymmetric carbon and so exists in two enantiomeric forms.

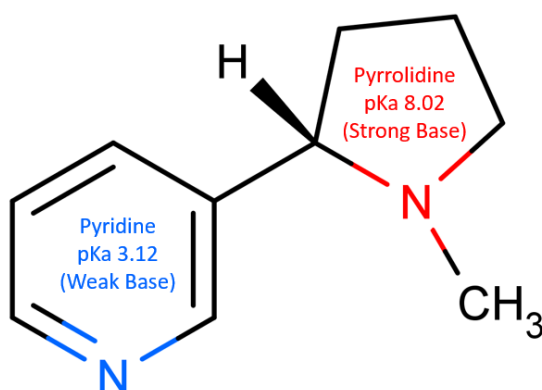


Figure 6-3 Nicotine chemical structure

Nicotine in tobacco is largely the levorotary (S)-isomer; only 0.1-0.6% of total nicotine content is (R)-nicotine (220). Unlike nicotine, the minor alkaloids anatabine, nornicotine, and anabasine and their N-nitroso derivatives are present to a substantial degree as the (R)-isomer (about 16, 20, and 42%, respectively) in tobacco products (220). Although nicotine (NIC) has a simple chemical structure, it undergoes an extensive biotransformation in the body and produces many metabolites. The presence of both aromatic and aliphatic carbon and nitrogen atoms on the nicotine structure provide many sites for different metabolic reactions.

<sup>6</sup> the **IUPAC nomenclature** of organic chemistry is a systematic method of naming organic chemical compounds as recommended by the International Union of Pure and Applied Chemistry (**IUPAC**).



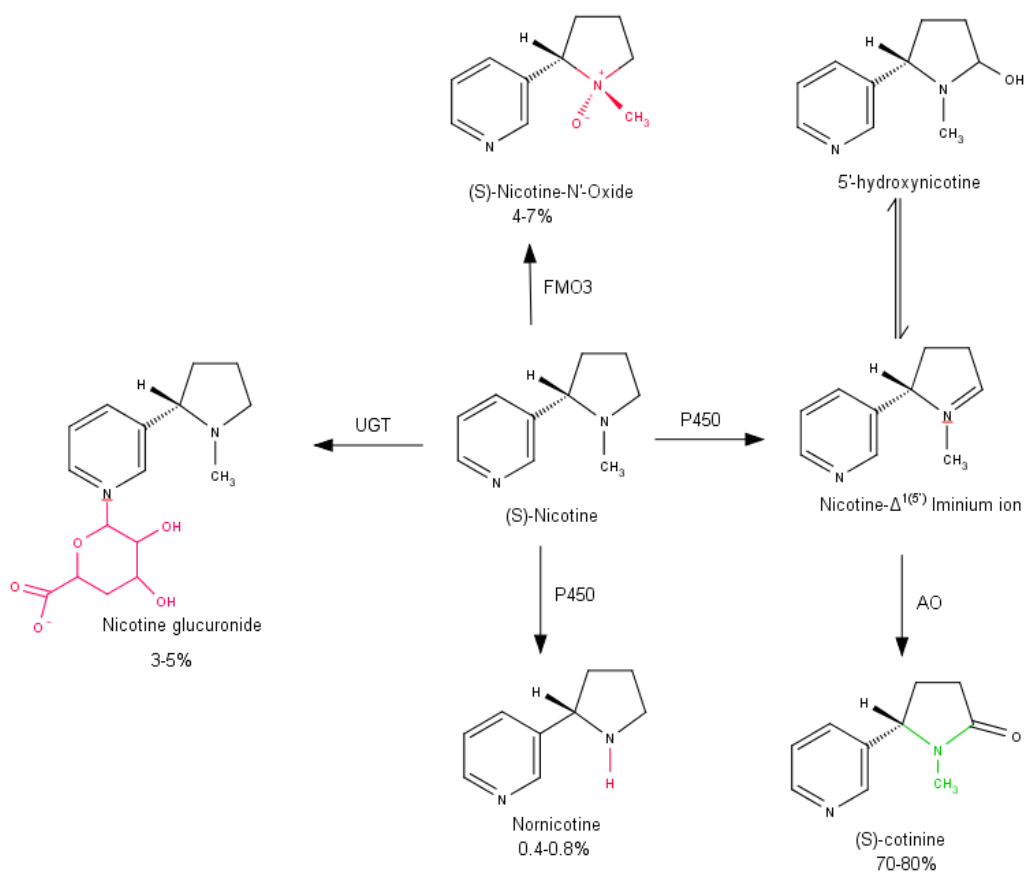
## 6.6 Nicotine Pharmacology in Humans

### 6.6.1 Absorption

After a puff, nicotine is extracted from burning tobacco and carried proximally on tar droplets, which are inhaled. When tobacco smoke reaches the small airways and alveoli of the lung, nicotine is rapidly absorbed. This is due to the huge surface area of the alveoli. Moreover, the dissolution of nicotine in the fluid (pH 7.4) in the human lung facilitates transfer across membranes. Shortly after a puff, high levels of nicotine reach the brain in 10-20 s (260).

### 6.6.2 Metabolism

Nicotine is mainly transformed in the liver. The most important metabolite of nicotine in humans is cotinine. Quantitatively, approximately 70 - 80% of nicotine is converted to cotinine. This conversion involves two steps; firstly, nicotine is converted to the nicotine- $\Delta 1'$  (5')-iminium ion, by CYP2A6, which is further transformed into cotinine by the aid of cytoplasmic aldehyde oxidase (AO) enzyme. The second most abundant nicotine metabolite is nicotine N'-oxide (NNO) (4-7%). The formation of this metabolite is mediated by flavin-containing monooxygenase 3 (FMO3) enzyme. In humans, the 1'-(S)-2'-(S)-trans-isomers are usually formed. Approximately 0.4-0.8% of nicotine is biotransformed into nornicotine by oxidative N-demethylation. Nicotine glucuronides (phase II metabolites) are formed by methylation of the pyridine nitrogen resulting in the nicotine isomethonium ion and glucuronidation. This reaction is catalysed by uridine diphosphate-glucuronosyltransferase (UGT) enzyme(s) producing (S)-nicotine-N- $\beta$ -glucuronide. About 3-5% of nicotine is converted to nicotine glucuronide and excreted in urine in humans (220,260). Figure 6-4 shows the main metabolic pathways of nicotine.



**Figure 6-4 Main metabolic pathways of nicotine**

### 6.6.3 Distribution

Smoking is a unique form of drug administration in that entry into the circulation is through the pulmonary rather than the systemic venous circulations. The time interval between smoking and entry into the brain is shorter than after intravenous injection. Nicotine enters the brain quickly, but then brain levels decline rapidly as nicotine is distributed to other body tissues. Because it is lipid soluble, nicotine has a large distribution volume (2 to 3 L/kg) and readily permeates cell membranes.

### 6.6.4 Excretion

It is important to mention that the percentages reported in section 6.6.2 represent the primary metabolic conversion products only and do not represent the final proportions of excreted metabolites in urine. Only 5% of a dose of NIC is excreted

unchanged in urine within a 24-hour period. The main metabolites of nicotine in urine are 3'-Hydroxycotinine and its glucuronide conjugate and Nicotine-N'-oxide (NNO) which account for 40-60% and 4%, respectively. The quantities of all other known metabolites are less than 5%. Acidity of urine enhances nicotine excretion and has limited effect on cotinine excretion. The elimination half-life of nicotine in humans, ranges from 1 to 3 hours while cotinine has a much longer elimination half-life of 10 to 14 hours.

## 6.7 Applications for measuring hair nicotine concentrations (HNC)

By reviewing the literature, 49 papers were identified to report a usage for the measurement of hair nicotine concentrations (HNC). Based on the main aim of the work, it was possible to classify them into 4 categories as illustrated in the below Table 6-1.

**Table 6-1 Main applications of HNC in the literature**

Main category	Subcategory	References
Report on advances in analytical method(s)		(196,262-267)
Pharmacological study		(268,269)
ETS exposure-disease relationship		(239,244,270,271)
ETS exposure	Mixed-gender adults	(240,261,265,272-282)
	Women only	(283-285)
	Infants or children	(239,243,244,252,267,270,271,286-288)
	Infants or children with their caregiver	(241,242,289-296)
	Companion animals (pets)	(238,297)

Reporting advances in the analytical methods was the main aim of several papers. Simultaneous and sensitive methods for measurement of nicotine and cotinine in small amounts (1 mg) of human hair using LC-MS/MS using a guard column and without analytical column was reported by Ryu *et al* (264). Nicotine and cotinine were included in a LC-MS/MS simultaneous methods for 14 and 17 drugs of abuse in hair by Kronstrand *et al* (266) and Koster *et al* (196), respectively. Pichini *et al* reported an attempt to develop hair reference material for nicotine and cotinine and evaluated different hair treatments and extraction procedures (262). Sporkert *et al* developed a solid-phase micro-extraction (SPME) method for a number of organic compounds including nicotine with a 1 ng/mg limit of detection (263). Karačonji *et al* optimised the SPME method that Sporkert *et al* started and focused on analysis of nicotine in hair and achieved a lower limit of detection at 0.02 ng/mg (267).

Another reason for quantifying nicotine, with/without its metabolites, in hair is to investigate long-term ETS exposure-associated diseases and possible links among groups of people. Groner *et al* measured hair nicotine concentrations in 145 subjects (9 to 18 years old) to investigate the impact of ETS exposure on cardiovascular status during childhood (244). Collaco *et al* investigated hair nicotine levels in 117 children with bronchopulmonary dysplasia (271).

Another reason for measuring HNC is to assess the pharmacology of nicotine in a selected group of subjects. Klein *et al* published a paper that looked into the changes in nicotine metabolism in 28 pregnant women who self-reported a steadily smoking behaviour during the whole gestational period (268). The paper reported an increase in nicotine and cotinine metabolism during pregnancy. Apelberg *et al* examined racial variations in HNC among 103 daily tobacco smokers. The authors concluded that, under controlled smoking conditions, black smokers have considerably higher nicotine levels in their hair than white smokers. The most dominant application for measurement of hair HNC is to measure ETS exposure and assess the utility of hair nicotine and/or its metabolites to discriminate active from passive smokers. Validity and acceptability of HNC for ETS exposure were examined by comparing the concentrations with the self-reported, or parental-reported use in cases involving children using a questionnaire. This category can

be further divided into 5 subcategories based on the different subjects. This will be covered in the following 5 sections.

### **6.7.1 ETS exposure studies employing mixed-gender adult subjects**

To assess ETS exposure, Jones *et al* measured air nicotine in 240 bars and nightclubs in 24 cities, and found elevated levels of nicotine in non-smoking and smoking workers' hair (n=625) (280). Similarly, Iglesias *et al*, evaluated the contribution of occupational against non-occupational ETS exposure to overall HNC in non-smoking bar and restaurant employees from Santiago, Chile (281). They concluded that working hours was the major determinant of hair nicotine concentrations in non-smoking employees in venues where smoking is allowed. In addition to air and hair nicotine, Agbenyikey *et al* measured the fine fraction of particulate matter (PM<sub>2.5</sub>) to assess ETS exposure in selected hospitality venues in Ghana (279). The authors found unacceptably high levels of ETS in public places where smoking was allowed. The concentrations of nicotine in air and non-smoking employee's hair were strongly and positively correlated with PM<sub>2.5</sub> concentrations in the air. To compare ETS exposure, biomarker concentrations found in different biological fluid and hair, Kim *et al* compared nicotine levels in hair with cotinine levels in saliva samples collected from the same subjects (282). Hair nicotine concentrations were found to be a more useful biomarker for the assessment of long-term exposure to tobacco, while, on the other hand, salivary cotinine concentrations were better reflecting recent tobacco exposure. Toraño *et al* determined simultaneously nicotine, cotinine and thiocyanate in urine, saliva and hair of 44 subjects (276). The authors were able to correctly classify 93.2% of active smokers from passive smokers by a combination of these three biomarkers using discriminant analysis. Polycyclic aromatic hydrocarbons (PAHs) are carcinogens formed as by-products of incomplete combustion of organic material. As PAH is not a specific indicator of ETS exposure, Appenzeller *et al* developed a simultaneous method to determine nicotine and twelve mono-hydroxy-PAHs in human hair. The work aimed to assess tobacco smoke contribution in PAH exposure (265). The method presented in their work is a useful tool for the accurate biomonitoring of chronic exposure to PAH and correct identification of the sources of exposure.

### 6.7.2 ETS exposure studies employing women only

Hair nicotine is an excellent tool for assessment of cumulated exposure to tobacco smoke exposure over a long time period. Several studies employed mother hair to test for nicotine concentrations, as an indicator of exposure during pregnancy, and then correlated these to their neonatal outcome. Using hair as a sample of choice, the effect of maternal tobacco smoke exposure, through active or passive smoking, on preterm birth, foetal growth and/or birth weight was assessed in four different articles (283-285,298). All four studies confirmed the finding of studies that were performed using different biological matrices and agreed that the exposure from active and, to lesser extent, passive smoking is closely associated with infants being small for gestational age, low birth weight births, and increases risk of infant health problems.

### 6.7.3 ETS exposure studies employing infants or children

Children, in particular, seem to be the most vulnerable population to be affected by ETS exposure. It is believed that the exposure to ETS considerably contributes to morbidity and mortality amongst children (299). As a result, many studies focused on assessing levels of exposure in children. Rasoloharimahefa-Rasamoela *et al* conducted a prospective cohort study to determine the ETS exposure among 318 children (aged 10-11) in Brussels (288). Their findings indicated that about 2-3% of children were active smokers despite the fact that they did not declare themselves as such, 53.4% of the children who did not actively smoke or live with a smoker had hair nicotine concentrations ranging from 0.63 ng/mg to 7.91 ng/mg. Similarly, Woodruff *et al* measured nicotine and cotinine in hair samples from 143 Latino children to assess their exposure to tobacco smoke (270). This study suggested that hair nicotine may be a more valid and practical biological measure than hair cotinine. Sørensen *et al* quantified the nicotine and cotinine concentrations in hair and plasma of 411 infants and compared the data with parentally reported levels of exposure (243). The authors concluded that the association between ETS exposure and nicotine in hair is the strongest association even at relatively low exposure levels.

#### **6.7.4 ETS exposure studies employing infants or children with their caregiver**

Smoker parents (or primary caregivers) are usually the main source of long-term systemic tobacco exposure to children. Many studies tested either one or both parents with their infant or child. Seong *et al* examined the contribution of paternal smoking to maternal and neonatal ETS exposure during pregnancy. This was achieved by testing hair samples from sixty three trios over two years (295). The study findings indicated that paternal smoking inside the home leads to significant foetal and maternal exposure to ETS. Similar conclusions were drawn by Chan *et al* in Hong Kong. In Chan's study, they looked into the exposure of the mother and children in families with a smoking father to assess the effectiveness of a newly-enforced smoke-free policy. Parents and their infants (<2 year of age) were recruited by Tzatzarakis *et al* to assess the validity and usefulness of hair nicotine-cotinine evaluation as a biomarker for monitoring ETS (296). The study findings identified the use of hair samples as an effective method for assessing exposure to tobacco. Maternal active or passive smoking during pregnancy is associated with foetal risk. Many studies looked into the levels of exposure in pregnant mothers and compared these with levels in newly-born babies' hair (241,289,290). In a multicountry study (31 countries), hair samples from 2480 women and children, potentially exposed to ETS, were analysed for nicotine. The study reported an increased risk of premature death and disease from exposure to ETS among women and children living with active smokers.

#### **6.7.5 ETS exposure studies employing the companion animals (pets)**

ETS exposure as a results of passive smoking was investigated using fur samples from companion animals (pet dogs) only in one paper by Bawazeer *et al* (238). The limited number of dogs (15 dogs) was a major limitation, however, the levels of biomarkers detected in the dog's fur gave a clear indication that companion animals fur could be a promising alternative to human hair to monitor long-term cumulative ETS exposure. Bertone *et al* carried out a study to evaluate whether exposure to ETS may increase the risk of feline malignant lymphoma in 80 cats with malignant lymphoma and 114 controls (258). The study findings indicated

that duration and quantity of exposure to ETS is positively associated with the risk of malignant lymphoma in cats.

## **6.8 Summary of experimental methods for the analysis of NIC and COT in hair**

### **6.8.1 Hair collection**

According to the Society of Hair Testing (SOHT) guidelines for drug testing in hair, the posterior vertex region of the head, as close as possible to the scalp, is the preferred region due to the uniformity of growth rate. Many studies that targeted hair nicotine reported collection of the samples from this region (196,241,267,268,270,278,284,285). Other areas have been reported. Zahlsen *et al* reported hair sampling from the borderline between hair and the naked skin behind each ear (274). This site, according to authors, has sufficient contact with surrounding air to measure the exposure.

### **6.8.2 Hair storage**

After collection, hair samples can be stored at room temperature for years within closed envelopes without loss or degradation of hair nicotine. Hertting *et al* identified nicotine in hair samples from Egyptian Mummies while Musshoff *et al* determined nicotine in hair samples of pre-Columbian mummies (300,301).

### **6.8.3 Hair mass**

The SOHT guidelines do not recommend a specific hair mass for analysis, however, as a rule of thumb, it is always recommended to use as small hair sample as possible. This is compromised sometimes by the method sensitivity. In the reviewed literature, researchers reported different hair mass ranging from a few milligrams (243,264,287,295) to 100 mg (273). In fact most of studies reported hair mass in the range of 10-50 mg (196,238,267,276,277). The employed analytical method and recruited subjects may dictate the hair mass volume. Lower hair volume was reported when RIA only was used (241,270). Jacqz-Aigrain reported collecting 50-80 mg maternal hair and only 5-10 mg neonatal hair (290).



#### 6.8.4 Hair washing

The first step after sample collection is the washing step to remove external contamination. Indeed, the question of whether the hair should or should not be washed, especially in case of ETS exposure studies, remains an issue. In general, it is preferable to include a washing step if the experiment is designed to measure inhaled exogenous nicotine or metabolites. In contrast, a washing step is not necessary when active smoking is excluded and the aim of the study is to measure the nicotine from ETS whether inhaled or deposited on the outer surface of hair. It is becoming common practice to refer to hair levels of nicotine and cotinine from unwashed hair as total nicotine and total cotinine. A range of washing solvents, including, water, acetone, methanol, dichloromethane, and detergent washes has been reported to be used to wash nicotine from the hair (238,241,252,263,270). Haley and Hoffmann compared the efficiency of acetone and hexane for hair washing. Dichloromethane (DCM) is believed to be a very effective solvent for washing nicotine from hair compared to a range of solvents (262). DCM has been reported as the washing solvent for nicotine from the hair in many studies (243,252,277,287,292,293,295). When washing is essential, it is good practice to retain the washings for analysis, if needed.

#### 6.8.5 Hair digestion

Following hair washing, the next step is to remove the incorporated drugs out of the keratinous matrix. Even if the research aims to investigate the ETS exposure only, it is important to get access to the drugs inside the hair. The deposition of drugs that are smoked, such as marijuana and nicotine from atmosphere could also be a possible route of incorporation into hair (302-305). Several laboratory methods have been developed to get access to the analytes of interest in hair, namely alkali or acid hydrolysis or extraction with organic solvents (mostly used methanol, hexane, acetone). Basic extraction using sodium hydroxide (NaOH) is favourable when substances (like THC, nicotine, antidepressants) are stable in aqueous NaOH. It is also the most reported method in the literature for extraction of nicotine from hair (252,262,264,265,267,273,274,287,293,297,298). However, Stout *et al*, based on their experiment, claim that sodium sulfide digestion of hair is a more efficient method of recovery of base-stable drugs from hair than the commonly used sodium hydroxide digest (306). Moreover, Bawazeer *et al*

compared the efficiency of alkaline and methanol for extraction of nicotine and its metabolite from dog's fur and concluded that the levels of nicotine obtained by both methods were found to be comparable (238).

#### 6.8.6 Extraction (sample clean-up)

Following hair digestion, some form of further cleaning is usually required. The sample preparation could be as simple as "dilute (or centrifuge) and shoot" or more complex such as liquid-liquid extraction (LLE), solid-phase extraction (SPE) or solid-phase microextraction (SPME). Several methods have been described for the analysis of nicotine and its metabolite in hair. Many methods describe an extensive sample preparation by incubating the hair sample followed by LLE (261,272-274,277,287,293,295,298), SPE (252,262,278,287,297) or SMPE (263,267). The selection of extract cleaning method is dictated by many factors including employed digestion method and analytical instrumentation. Using alkaline hydrolysis will produce a very dirty extract which requires an extensive cleaning step before injection into a system such as GC-MS or LC-MS. In previous studies, diethyl ether was used most often for the extraction of hair nicotine (261,272-274,287,293,295,298). However, Chetianukornkul *et al* compared the extraction recoveries of diethyl ether and dichloromethane and reported better recoveries with the DCM (277).

#### 6.8.7 Instrumentation

Concentrations of nicotine and its metabolites have been determined by using a range of techniques, including gas chromatography (GC) (coupled with nitrogen-selective detection (NSD) and mass spectrometry(MS)) and High Performance Liquid Chromatography (HPLC) coupled with Ultraviolet (UV) detection, electrochemical detection (ECD) and MS. Gas or liquid chromatography with mass spectrometry is most commonly used due to its advantage of simultaneous measurement of nicotine and its metabolites with deuterium labels used as internal standards. Table 6-2 summarises the instrumentations used in the included 49 papers.

**Table 6-2 Analytical techniques reported in the literature for analysis of nicotine and its metabolites in hair matrices**

Chromatography	Detector	References
Gas chromatography	NSD (NPD)	(271,272,279,291,294)
	MS	(243,261,263,265,267,269,273-276,280-283,292,293)
	MS/MS	(307)
HPLC	UV	(252,262)
	ECD	(239,244,284,287,308)
	MS	(238,277,296,297)
	MS/MS	(196,264,266,278,295,298)
Radioimmunoassay	NA	(240-242,268,270,285,286,290)
No method reported		(288,289)

There have been several studies investigating the concentrations of NIC and COT in hair after passive exposure. The range of concentrations detected in children hair is compared against the concentrations obtained in this thesis and shown in detail in section 8.8.1.

## 6.9 Aims

### 6.9.1 Method development for analysis of NIC and COT in hair

The main aim of this project was to develop an analytical method for the quantification of NIC and COT in hair. The method developed for hair was used to analyse fur samples from dogs to establish the extent of exposure to ETS. There are many methods in the literature for the analysis of nicotine and cotinine in human hair, but there is only one method that has been applied to dogs' fur specimens. Only one LC-MS method has been applied to dogs fur specimens and this did not achieve the sensitivity required for testing levels of NIC and COT in passively exposed subjects to ETS (238). A method was required that provided the acceptable selectivity and sensitivity for all of the chosen analytes. It needed to be relatively quick to enable efficient throughput in the laboratory. Optimised methanolic extraction with centrifugation provides a quick and simple method for the extraction of nicotine and cotinine from dogs' fur samples. LC/MS-MS is an ideal instrumental technique for quantification of nicotine and cotinine in fur

specimens as it provides the required sensitivity (low pg/mg concentration range) and selectivity by eliminating unwanted background matrix interferences that are common in animal fur specimens.

### **6.9.2 Application of method to fur samples from companion pet dogs exposed to ETS**

There is limited data available for concentrations of NIC and COT detected in pet dog fur and the effect of ETS exposure on pets in general. It is not well-known whether pets' fur can be used to measure the long-term ETS exposure and mimic the levels that children are exposed to or not. Unlike human hair, dogs' fur is exposed only to limited hair treatments. This can be considered as an advantage for dog fur over human hair. The extensive range of human hair treatments and washing is known to affect the concentrations and complicate results interpretation. The developed and validated method was applied to analyse 66 pet dog fur samples that were collected and submitted for analysis from the School of Veterinary Medicine, University of Glasgow. This included a cross-section of cases that were representative of the cases normally received for treatment in the veterinary clinic. Analysis for NIC and COT was carried out to investigate the ETS exposure. Analysis of these cases was conducted to compare the results with the owner-reported ETS exposure levels in a questionnaire.

### **6.9.3 Cut-off values of hair nicotine and cotinine in companion animals fur to discriminate the degree of exposure to tobacco smoke**

As dogs have comparable hair growth rate to humans (297), it is expected that HNC should therefore mirror a similar time frame of ETS exposure. Our measurements on dog's fur exposed to various degrees of ETS indicate that cut-off limits for hair nicotine between heavy and light exposure may exist. Earlier studies proposed cut-offs to distinguish active and passive smokers. Zahlsen *et al* indicated that a cut-off limit probably exists somewhere between 2 and 5 ng/mg (274) . Kintz *et al* indicated a cut-off limit at 2 ng/mg (273).

## **Chapter 7 Method Development and Validation of a Method for the Analysis of NIC and COT in Hair matrices**

### **7.1 Introduction**

Nicotine (NIC) and cotinine (COT) are two of the most abundant naturally occurring tobacco-specific alkaloids. Various analytical methods have been described in the literature for analysis of nicotine and its metabolites in hair matrices. Different sample preparation procedures and instrumentation were reviewed in chapter 6. This chapter will focus on the method development and optimisation and validation carried out as part of this study. Chromatographic separation of nicotine and cotinine was achieved using a hydrophilic interaction chromatography (HILIC) column. Identification was achieved on the MS detector. Optimal MS parameters were obtained from direct infusion. The aim of this project was to develop and validate a method for the determination of nicotine and cotinine using liquid chromatography tandem mass spectrometry (LC-MS/MS). An optimised methanolic extraction method was employed. Validation was carried out according to guidelines of Scientific Working Group for Forensic Toxicology (SWGTOX). The validated method was then applied to the analysis of sixty-six fur samples to investigate dogs' exposure to indoor and outdoor ETS.

### **7.2 Materials and Methods**

#### **7.2.1 Chemicals and reagents**

Cerilliant certified reference materials for s(-)-nicotine (1 mg/ml), (-)-cotinine (1 mg/ml), and internal standards, (+\)-nicotine-d4 (100 µg/ml), (+\)-cotinine-d3 (100 µg/ml) and formic acid were obtained from Sigma Aldrich Company Ltd., Dorset, UK. Acetonitrile, methanol and dichloromethane (DCM) (HPLC grade) were obtained from VWR International Ltd, UK. Captiva Premium Syringe PES Filters (polyethersulfone (PES) membrane, 15 mm diameter, 0.2 µm pore size, LC/MS certified), were obtained from Agilent Technologies, UK. Chromacol Environmental Vials and 7 mL silanised vials were purchased from Thermo Fisher Scientific, UK. Ultrapure deionised water was obtained from the in-house Millipore purification system.

### 7.2.2 Instrumentation

Analysis was carried out using an Agilent LC-MS-MS triple quadrupole G6430A mass spectrometer equipped with an Agilent 1200 series autosampler, A quaternary pump SL with degasser and thermostatted column compartment was used. Positive electrospray ionization (+ESI) was used and the MS operated in multiple reaction monitoring mode (MRM). Analytes of interest were separated on a SeQuant ZIC-HILIC<sup>®</sup> column (150 x 4.6 mm, 5 µm), protected by a guard column with identical packing material (20 x 2.1 mm) both obtained from Merck Millipore, UK.

### 7.2.3 LC-MS-MS Operating Conditions

The optimal conditions were achieved using a nebulizer pressure at 15 psi, a capillary voltage of 4,000 V, nitrogen gas heated to 350 °C and delivered at 11 L/min. The column temperature was maintained at 40 °C. Gradient elution was employed using a mobile phase consisting of A: deionised water with 0.1% formic acid (FA) and B: Acetonitrile with 0.1% FA at a flow rate of 0.5 mL/min. The total run time was 30 min. The gradient mobile phase system started at 60:40 A/B increasing to 40:60 A/B within 10 min. The percentage organic content was then decreased to 60:40 A/B in 0.2 minutes and maintained for 19.8 min in order to condition the column before the next injection. LC and MS source parameters are summarized in Table 7-1. Optimal MS parameters were obtained by direct infusion of both drugs of interest and their deuterated ISTD to the electrospray ionisation (ESI) source in positive mode. The infusion solution consisted of 50/50 (v/v) of aqueous and organic mobile phase at a concentration of 1 µg/ml for all analytes. Following European Union Decision 2002/657/EC, 5 identification points were achieved by monitoring 2 precursor ions, each with 1 daughter ion for each analyte. The retention time ions ratios for both analytes are additional identification criteria that were monitored. Table 7-2 summarises the optimal MS transitions and parameters.

**Table 7-1 Summary of LC and Ion Source Parameters**

LC Parameters	
Column	SeQuant® ZIC®-HILIC (5µm,200Å) PEEK 150 x 4.6 mm with guard column of the same packing material
Mobile phase	A: dH <sub>2</sub> O with 0.1% FA and B: ACN with 0.1% FA 10 mins: 60:40 A/B → 40:60 A/B 0.2 mins: 40:60 A/B → 60:40 A/B 19.8 mins: maintained at 60:40 A/B
Flow rate	0.5 mL/min
Injection volume	20 µL
Column Temperature	40°C
Run Time	30 minutes
Mass Spectrometry Parameters	
Operating mode	ESI-positive mode
Gas temperature	350°C
Gas flow	11 L/min
Nebulizer Pressure	15 psi
Capillary Voltage	4000 V
Scan mode	MRM

**Table 7-2 MRM transitions of NIC, COT, NIC-d4 and COT-d3**

Drug	Precursor Ion (m/z)	FV*	Abundance of precursor ion	Product ion (m/z)	CE#	Abundance of product ion
s(-) Nicotine, MW: 162.23	163.00	100	35000	130	10	4000
				117	20	6500
				106	20	2500
(±)-Nicotine-d <sub>4</sub> , MW: 166.20	167.00	110	60000	136.2	10	3200
				110.10	20	1500
(-)-Cotinine, MW: 176.22	177.10	130	20000	98.00	20	5000
				80.10	30	15000
(±)-Cotinine-d <sub>3</sub> , MW: 179.19	180.00	115	28000	101.1	20	5000
				80.10	30	16000

\*Fragmentor Voltage

#Collision Energy

## 7.2.4 Preparation of Dogs' Fur Sample

### 7.2.4.1 Blank fur

It is recommended by SWGTOX to matrix match calibration standards and QCs to the type of specimens that are being analysed. Blank fur samples used for development and validation of the method were collected from 21 dogs of non-smoker owners and reported to be living in tobacco-free houses with no active smoker. Samples were wrapped in aluminium foil and stored in the hair collection envelopes at room temperature within forensic medicine and science (FMS). The scissors were cleaned between participants, using methanol, and then dried with a paper towel before cutting the next sample. The fur samples were transferred from the collection envelope with clean tweezers. The used scissors and tweezers were cleaned with methanol before and after each sample. There was no contact between fur samples and hands during the sampling procedure. All 20 blank fur samples were pretested for the presence of nicotine and cotinine before pooling the fur to create a large analyte-free control. Table 7-3 summarises the results of blank fur screening tests. Sample analysis was carried out using the final optimised procedure that will be described in this chapter. Samples were designated positive if the signal to noise ratio was greater than 10 for all transitions. Samples were designated as positive at a concentration less than the limit of quantitation (<LOQ) where the measured concentration was less than LOQ but all transitions were present. All samples that tested positive or less than LOQ are highlighted in red in Table 7-3 .and Although all blank fur samples had a signal for the nicotine precursor transition, they were considered negative when one or both product transitions had a signal to noise ratio of less than 3.



**Table 7-3 Screening results of 21 blank fur samples**

Blank ID	Results		Blank ID	Results	
	NIC	COT		NIC	COT
B1	NEG	NEG	B12	NEG	NEG
B2	<LOQ	NEG	B13	NEG	NEG
B3	NEG	NEG	B14	<LOQ	NEG
B4	NEG	NEG	B15	<LOQ	NEG
B5	<LOQ	NEG	B16	<LOQ	NEG
B6	NEG	NEG	B17	NEG	NEG
B7	NEG	NEG	B18	NEG	NEG
B8	POS	POS	B19	POS	NEG
B9	NEG	NEG	B20	<LOQ	<LOQ
B10	NEG	NEG	B21	POS	<LOQ
B11	<LOQ	NEG			

The homogenized pools stocks were prepared by pooling negative blank fur specimens shown in Table 7-3. The pooled blank fur control was stored in 20 mL capped Chromacol environmental vials at room temperature to be used for preparation of calibrator and quality control specimens.

#### 7.2.4.2 Fur washing

20 mL Chromacol environmental vials with screw caps were used to store and weigh the fur prior to carrying out the wash procedure and extraction. The utilised washing procedure involved both organic solvent and aqueous solutions. As dichloromethane (DCM) is considered to be a suitable wash solvent because it does not swell the fur, 2 mL DCM was used to wash the fur first with 15 minutes of sonication, this step was repeated if needed. The second wash was achieved using 2 mL deionised water with 3 minutes sonication followed by 2 mL methanol with less than a minute sonication to remove water residues and accelerate the drying process. Longer sonication with methanol was avoided to prevent fur swelling. After washing, fur samples were dried overnight in an oven at 40°C. The dry washed fur samples were cut with a pair of scissors into 1-2 mm pieces. 30 mg of that was weighed and extracted on the day of analysis. The above described wash method was used to wash blank samples involved in nicotine and cotinine method validation and extraction recovery studies.

For optimisation of nicotine and cotinine release from hair into extraction solvent, and hence incubation time, 30 real case samples of good sample size were used. The wash procedure involved using the extraction solvent itself only (methanol with 0.1% formic acid) with sonication for 15 minutes at room temperature. Longer sonication with methanol was avoided to prevent fur swelling which could actively remove the drugs from inside the fur. For measurement of total nicotine and cotinine concentrations in the 66 case samples, no washing was involved.

## **7.2.5 Preparation of Solutions**

### **7.2.5.1 Preparation of Stock and Working Standard Solutions**

Separate stock solutions of nicotine and cotinine (100 µg/mL) and nicotine-*d*3 and cotinine-*d*4 (10 µg/mL) were prepared from purchased stock solutions by transferring 1 mL to a 10 mL volumetric flask and making up to the mark with methanol, except cotinine-*d*4 which was received and hence diluted in acetonitrile. The prepared stock solutions were then stored at -20 °C. Two sets of working solutions of the standards and internal standards were prepared at a concentration of 1 µg/mL (set1) and 100 ng/mL (set2) by dilution of the stock solutions using methanol. The prepared working solutions were stored at -20 °C.

### **7.2.5.2 Preparation of Quality Control (QC) Samples and Calibrators**

Quality control standards (QCs) were prepared and analysed in addition to calibration standards and samples to ensure that accurate results were being obtained for the specimens. For extraction recovery studies, QC materials preparation is described in detail for each experiment in section 7.5. For the method validation and case samples analysis, QC material was prepared in blank fur screened negative for nicotine and cotinine at concentrations of 0.01, 1, and 5 ng/mg for both nicotine and cotinine. The quality control materials were freshly prepared on the day of analysis. Ten-point calibration curves at concentrations of 0, 0.01, 0.05, 0.1, 0.5, 1, 2, 5, 10 and 20 ng/mg were prepared. Table 7-4 and Table 7-5 summarise the preparation of the control materials for method validation and calibration levels.

**Table 7-4 Preparation of QC Materials for Method Validation and Case Samples Analysis**

QC points	Working solution (µl)	Extraction solvent (mL)	Fur (mg)	[ng/mg]
QC 1	15 (set2)	2	30 ± 5	0.05
QC 2	30 (set1)*	2	30 ± 5	1
QC 3	150 (set1)	2	30 ± 5	5

\*Internal standard concentration

**Table 7-5 Preparation of Calibration Curves for Nicotine and Cotinine**

Cal. levels	Working solution (µl)	Extraction solvent (mL)	Fur (mg)	[ng/mg]
Blank	0	2	30 ± 5	0
Level 1	3 (set2)	2	30 ± 5	0.01
Level 2	15 (set2)	2	30 ± 5	0.05
Level 3	30 (set2)	2	30 ± 5	0.1
Level 4	150 (set2)	2	30 ± 5	0.5
Level 5	30 (set1)*	2	30 ± 5	1
Level 6	60 (set1)	2	30 ± 5	2
Level 7	150 (set1)	2	30 ± 5	5
Level 8	300 (set1)	2	30 ± 5	10
Level 9	600 (set1)	2	30 ± 5	20

\*Internal standard concentration

### 7.2.5.3 Preparation of extraction solvent

The extraction solvent (methanol with 0.1% formic acid) was prepared by adding appropriate volume of formic acid to methanol. For preparation of 100 mL, 100 µl of the concentrated formic acid was added into 100 mL volumetric flask and made up to 100 mL with methanol.

### 7.2.5.4 Preparation of mobile phase solutions

An acidic mobile phase consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile was prepared by adding 1mL of concentrated formic acid to a 1000 mL volumetric flask and making up to 1000 mL with deionised water or acetonitrile, respectively.

### 7.3 Nicotine and cotinine extraction from fur

Methanol is one of the most commonly used extraction solvents and is often supported by the use of an ultrasonic bath to reduce the extraction time. The extraction times with methanol are usually quite long due to the long incubation time that can take between 16 and 20 hrs (309,310). However, the use of an ultrasonic bath combined with a higher temperature has proven to shorten the extraction time up to 4 hrs (311,312). High temperatures can cause degradation of some substances (e.g. conversion of cocaine into benzoylecgonine (313)) and must be used with caution. Moreover, it has been reported that the acidification of the extraction solvent could improve the extraction efficiency of basic molecules such as amphetamine, cocaine and nicotine. Barroso *et al* reported a significant improvement in extraction efficiency of cocaine and its metabolite benzoylecgonine after acidifying methanol with hydrochloric acid (HCl) (313). It is possible that the acid prevents or decreases affinity of drug-protein binding in hair. The applicability of either adding heat or acid to the extraction solvent for nicotine is not known as it has not been reported in the literature to date. However, the widespread and frequently reported use of alkaline hydrolysis at high temperature (ranging from 60 to 90 °C) to extract nicotine and its metabolites from hair (see section 7.8) indicates that nicotine is thermally stable and will not degrade at temperatures lower than 60 °C. Therefore, 50°C heat was added during sonication. For acidification, nicotine is known to react with hydrochloric acid in water to form the water-soluble salt nicotine hydrochloride. Hence, acidification of the extraction solvent with HCl was found not to be appropriate. As an alternative approach, formic acid was used to acidify the extraction solvent. The chosen methanolic extraction conditions for nicotine and cotinine were 2 hrs with sonication at 50 °C. After extraction, the 2mL methanolic extract was then transferred into a clean vial for further cleaning before injection.

### 7.4 Justification of column selection

Method development started by using a mobile phase of (A) 0.1% formic acid in water and (B) 0.1% formic Acid in acetonitrile. A reversed-phase (RP) Phenomenex Gemini C18 (150 x 2.1 mm, 5 µm) column coupled with a C18 guard column (4 x 2.0 mm) was used first as it is one of the most commonly employed separation columns in forensic toxicology. An isocratic system with a high aqueous mobile

phase content (90%) was used first. The aim of this was to check whether nicotine and cotinine would interact with the C18 stationary phase of the column and for how long they would be retained on the column. Although the aqueous-rich mobile phase has poor elution strength, both drugs were eluted within one minute (void volume) after injection. A similar retention time was obtained when a higher organic solvent mobile phase content was used (30%B). Thus, the RP Gemini C18 column was replaced as it was unable to achieve acceptable chromatographic separation. As NIC is a basic polar drug, the Synergi Polar-RP (150 x 2.1 mm, 4  $\mu$ m) was installed and tested. Column testing was started using an isocratic system with a high solvent content. The goal of this was to assess the ability of the column to retain the drugs. It was found that both drugs were interacting with the stationary phase and eluted 3 minutes (Cotinine) and 7.8 min (Nicotine) after injection. The observed improvement in the sensitivity when using this column compared to C18 column is believed to be due to the elution using a high percentage of solvent (MS friendly). Despite the satisfactory retention of drugs of interest by this column, peak broadening was a problem and the column failed to achieve the minimal acceptable criteria for the peak shape chromatography. After reviewing the literature for the available analytical column options, hydrophilic interaction chromatography with a covalently bound zwitterionic functional groups (ZIC-HILIC) column was reported for analysis of NIC and its main metabolite (238). Furthermore, this column was reported to enhance sensitivity in mass spectrometry and is compatible with a wide range of buffers and organic solvents for injection.

#### **7.4.1 Hydrophilic interaction chromatography (HILIC)**

The abbreviation HILIC was first proposed by Alpert in 1990 (314). Hemström and Irgum published a review about HILIC in 2006 and reported a considerable increase in the number of publications on HILIC since 2003(315). HILIC method development started with an isocratic system using a mobile phase with high organic solvent content (B=80) as per column manufacturer recommendation. It was found that cotinine had a retention time of 7.8 minutes while nicotine's retention time was 16.9 minutes (See Figure 7-1(B)). As fur samples are complex and have the potential for more interferences, it was necessary to use a gradient system after elution of the analytes of interest to clean the column. With ZIC-HILIC columns, the aqueous mobile solvent is a stronger elution solvent than the aprotic organic

solvent. Hence, a cleaning ramp was added to the method starting from 90% organic content as per column manufacturer recommendations. It was found that cotinine eluted at 8.9 minutes while nicotine eluted at 14.5 minutes (See Figure 7-1 (A)). This method had a very long run time (40 minutes) due to the equilibrium time at the end. Another gradient system starting with 60% organic MP provided acceptable separation, better peak shape and reduced the run time to 30 minutes (See Figure 7-1 (C)). Removing formic acid from the mobile phase and replacing it with 2 mM ammonium acetate was found to have a significant negative impact on the peak shape and sensitivity.

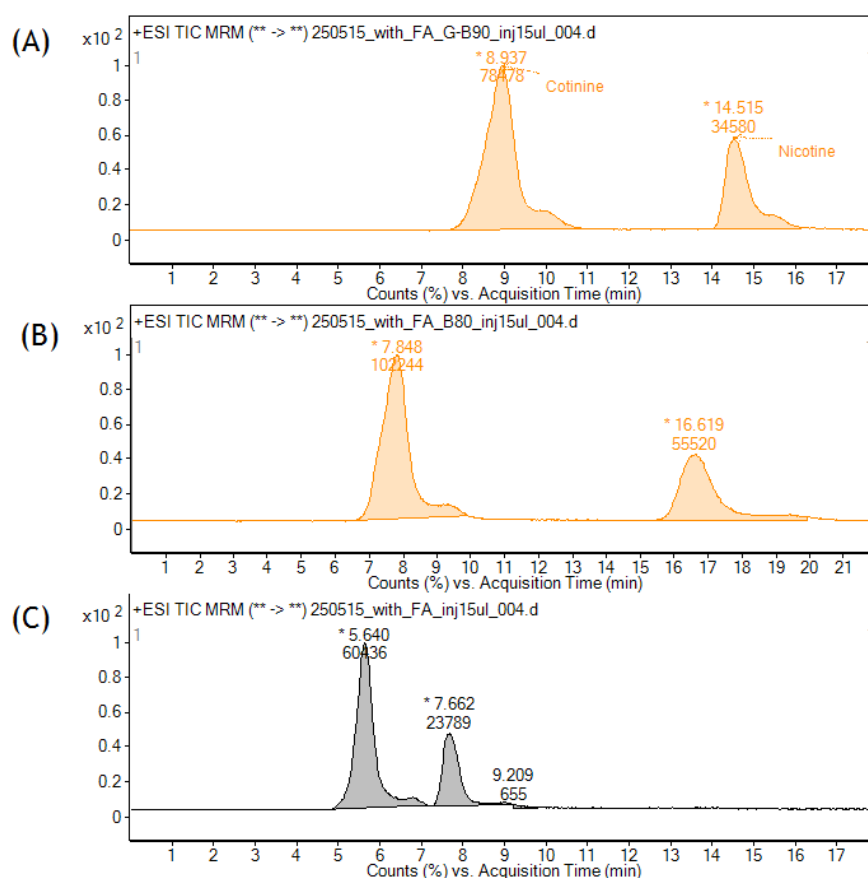


Figure 7-1 three chromatograms shows the effect of gradient system changes on drugs retention times and peak shape; (A) B=90%, (B) B=80%, (C) B=60%.

## 7.5 Evaluation of methanolic extract clean-up methods

### 7.5.1 Evaporation/reconstitution cycle

A recovery study was carried out to determine the amount of drug loss during evaporation. The main washing solvent (DCM) and extraction solvent (methanol) were included in the recovery study. 2 mL of washing and extracting solvents (no

fur used for this experiment) were spiked at three different concentrations (5, 30 and 200 ng/2mL) and evaporated to dryness under a gentle stream of nitrogen at 50 °C to accelerate the evaporation process. Each sample was reconstituted with 200 µl methanol with 0.1% formic. 30 ng (30 µl of 1 µg/mL (set1)) internal standard mix was then added after reconstitution. The signal obtained was compared to the response from a similar amount of drug in 200 µl methanol with 0.1% formic acid.

### 7.5.2 Filtration

30 mg samples of blank fur were spiked at three different concentrations of nicotine and cotinine (10, 30 and 120 ng/30mg) and were subjected to extraction with methanol containing 0.1% formic acid. To remove particulates, the extract was then filtered through Captiva Premium Syringe PES Filter. 30 ng (30 µl of 1 µg/mL (set1)) of deuterated ISTD nicotine-*d*3 and cotinine-*d*4 was added after filtration. Recovery was assessed by comparing the peak area ratio (drug/IS) from filtered samples to equivalent concentrations from spiked, unfiltered extraction solvent (2mL). Three replicates were extracted and averaged.

### 7.5.3 Centrifugation

Centrifugation was proposed as a cheaper alternative to the filtration. 30 mg samples of blank fur were spiked at three different concentrations of nicotine and cotinine (10, 30 and 200 ng/30mg). Spiked fur samples were combined with 2 mL extraction solvent and then centrifuged at 4000 rpm for 10 minutes. The supernatant was transferred to a clean vial and 30 ng of deuterated ISTD nicotine-*d*3 and cotinine-*d*4 was added. Similar to the filtration experiment, recovery after centrifugation was calculated.

## 7.6 Injection Volume Optimisation

As per column manufacturer instructions for care and use, 5 to 50 µl can be injected into a column with 4.6 mm internal diameter (ID) and the recommended injection volume is about 1% of the total column volume ( $V_m$ ). The column volume was calculated and three different volumes within the allowed range, 10, 20, 50

µl were tested. The criteria for selection of the injection volume were the sensitivity and peak shape.

## **7.7 Method validation**

The validation of the method was completed in accordance with the Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines. This included the determination of the limit of quantification (LOQ), limit of detection (LOD), interference, calibration model, carry-over, precision and accuracy, recovery, and matrix effects (172). Excel Windows 2010 data analysis was used to calculate the average, median and standard deviations. Regression and data analysis were carried out using Agilent MassHunter Workstation software (version: B.01.05).

### **7.7.1 Limit of detection (LOD) and Lower Limit of quantitation (LLOQ)**

The LOD and LOQ determination was carried out by spiking blank fur samples at decreasing concentrations within the expected range of detection sensitivity. The LLOQ and LOD were calculated using the reference materials approach in accordance with SWGTOX guideline (172). Three sources of blank matrix samples fortified at decreasing concentrations (0.01, 0.03, 0.05, 0.07, 0.1 ng/mg) were analysed in duplicate (two separate samples) for three runs. Regression analysis and signal to noise ratio (S/N) were calculated using the instrumental software (MassHunter). For both nicotine and cotinine, concentrations that yielded a reproducible response greater than or equal to three times the noise level of the background signal from the negative samples were assigned as the LOD, while concentrations that yielded a reproducible response greater than or equal to ten times the noise level were assigned as the LLOQ.

### **7.7.2 Calibration model (Linearity)**

Calibration model can be defined as the mathematical model that demonstrates the relationship between the concentration of analyte and the corresponding instrument response (316). The calibration model was investigated over the concentration range relevant for analysis of nicotine and cotinine in non-smokers on five different runs. Calibration curves were prepared by spiking 1.5, 3, 15, 30, 60, 150 and 300 ng amounts of nicotine and cotinine and adding 30 ng of nicotine-



*d4* and cotinine-*d3* into 30 mg of blank fur combined with 2 ml amounts of methanol with 0.1% formic acid.

### 7.7.3 Accuracy and precision

Precision and accuracy were measured using a calibration curve for the optimised method prepared with each run. Intra-day data were assessed by comparing data from within one run ( $n = 3$  for each QC). Inter-day validation data were obtained from analyses conducted on five different days. Pooled blank fur from five sources was spiked at three different concentrations (low 0.05 ng/mg, medium 1 ng/mg and high 5 ng/mg).

### 7.7.4 Matrix Effect

The change in response observed for a given concentration of analyte of interest in the presence of other sample components can be defined as matrix effect. Although fur is not a biological fluid, it is a biological tissue, when sonicated with methanol it will swell and release its internal component to the extraction solvent; moreover, dirt on the outer surface of the fur may contribute to the matrix effect as well. These components can either cause suppression or enhancement of the target analyte response. Matrix effect was assessed by comparing the response from spiked extracts to that from spiked clean extraction solvent at two different concentrations.

The matrix effect was assessed using the Post-Extraction Addition Approach. Two sets of samples were prepared at low (0.05 ng/mg) and high (5 ng/mg) concentrations. Set one consisted of neat standards injected six times to establish a mean peak area for each concentration. Set two consisted of three different matrix sources. Each matrix source was extracted in duplicate and the extract spiked with either the low or high concentration. The matrix effect was then calculated by averaging the area of each as follows:

$$\%ionisation\ enhancement\ or\ suppression = \left( \frac{\bar{X}\ Area\ of\ set2}{\bar{X}\ Area\ of\ set1} - 1 \right) * 100$$

**Equation 7-1 Ionization suppression or enhancement percentages**

A negative value indicates signal suppression whereas positive values suggest that some enhancement is occurring. According to SWGTOX guidelines, the acceptable limits for enhancement or suppression are  $\pm 25\%$ .

### **7.7.5 Carryover**

According to SWGTOX validation guidelines, carryover is defined as appearance of unintended analyte signal in samples after the analysis of a positive sample. Carryover was assessed by injecting extraction solvent blank following a sample that contained equivalent concentrations to the upper limit of quantitation (20 ng/mg) on five different runs.

## **7.8 Results and discussion**

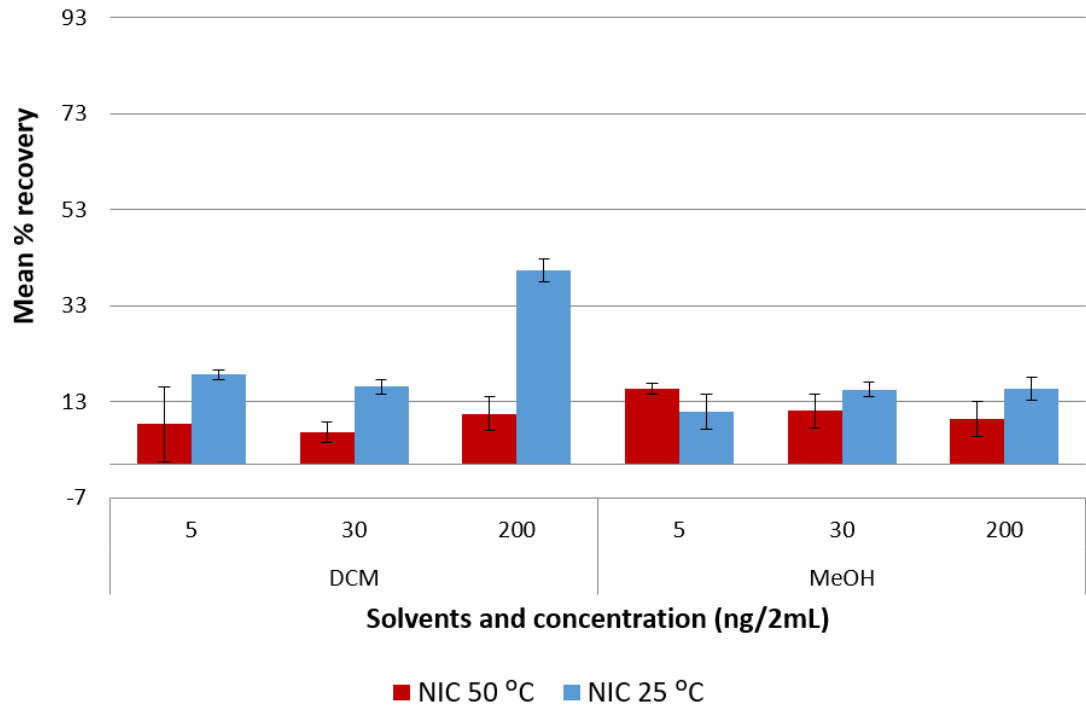
### **7.8.1 Evaluation of methanolic extract clean-up methods**

After methanolic extraction, it was necessary to clean-up the extract further. Although the extract was not, visually, too dirty, it was not suitable for direct injection. The initial plan was to evaporate the DCM washes and methanolic extract and reconstitute with a suitable aqueous solution to then undergo further clean-up using LLE or SPE to improve the analyte detection sensitivity. Filtration and centrifugation were later proposed as alternatives that will allow direct injection into ZIC-HILIC-MSMS. Recoveries and drug loss were monitored for all proposed methods. Three replicates for three levels low, medium and high were prepared, extracted and averaged as explained earlier in section 7.5.

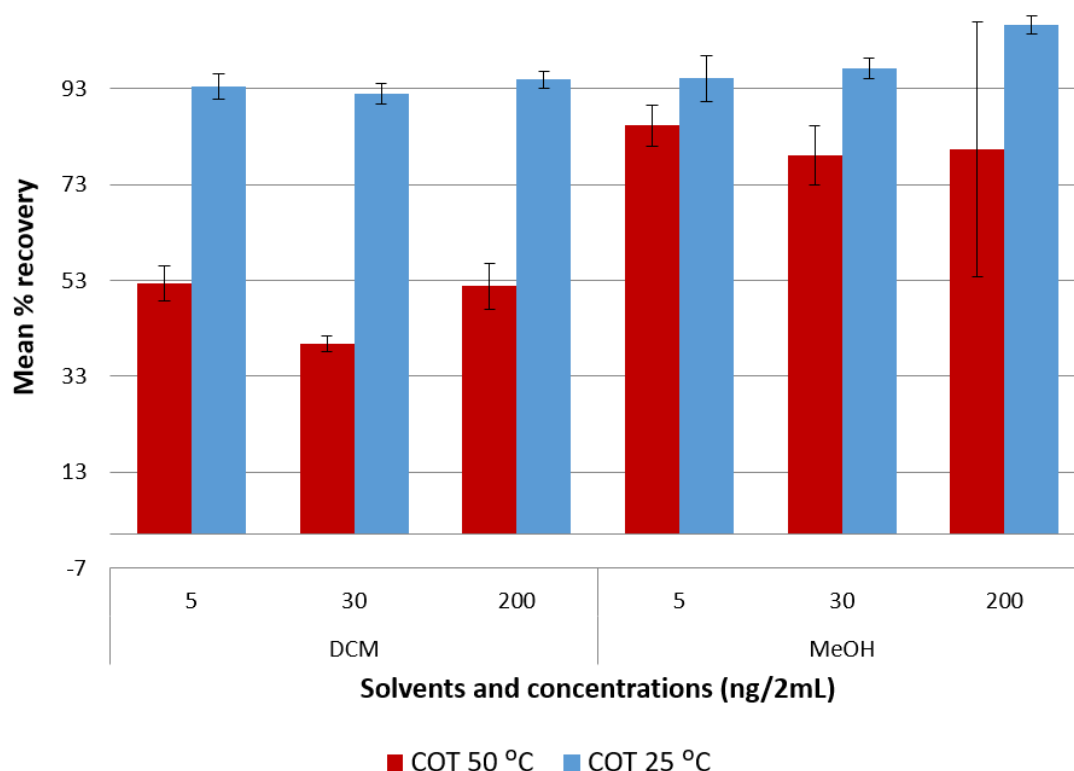
#### **7.8.1.1 Evaporation / Reconstitution Cycle**

It was observed that much lower responses for nicotine were obtained after evaporation in comparison with non-evaporated standards at equivalent concentrations. As illustrated in Figure 7-2 and Figure 7-3, more than 90% of COT was recovered from methanol and about 30-50% from DCM. For nicotine, poor %recovery was found from both solvents. As heat was a possible cause of drug loss during evaporation and resulted in low % recovery, the experiment was repeated and evaporation was carried out at room temperature (25 °C) to investigate further. Better results were observed at room temperature for the recovery of

COT from DCM and similar results at 50 °C for NIC (Figure 7-2). Nicotine is volatile and it is possible that it evaporates even at room temperature.

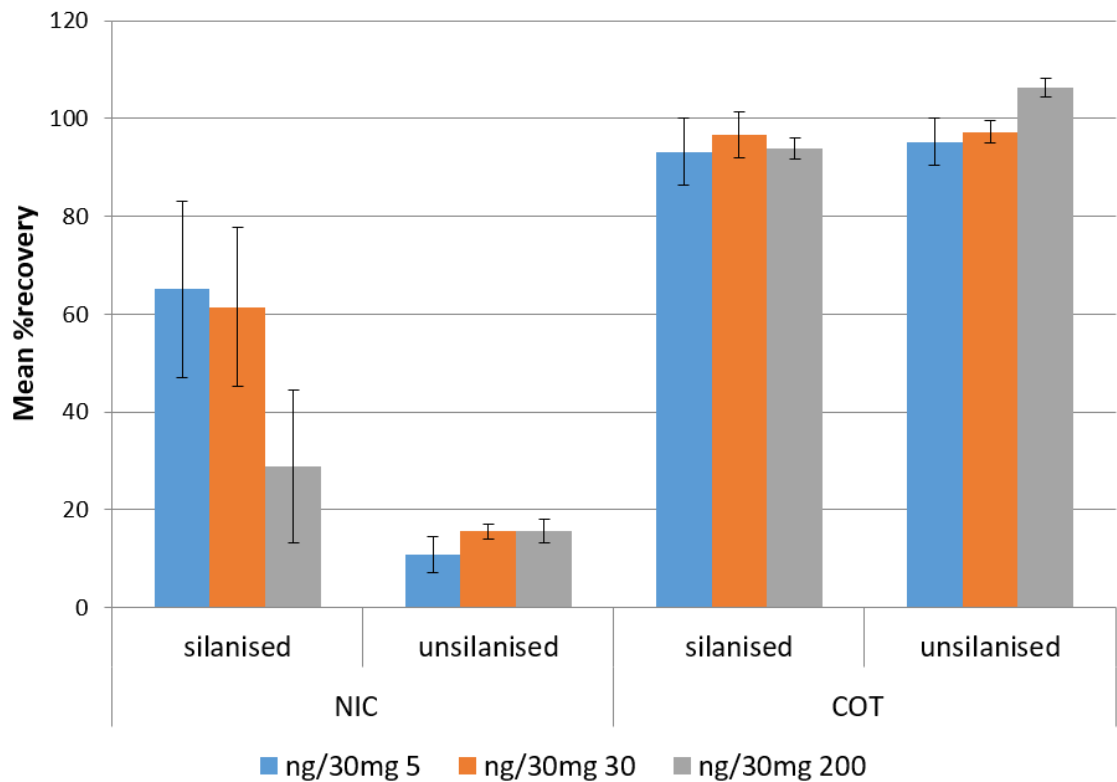


**Figure 7-2 Mean %Recovery ( $\pm$  1 s.d.) for nicotine (NIC) from dichloromethane (DCM) and methanol (MeOH) after evaporation and reconstitution 200  $\mu$ l methanol with 0.1% formic acid**



**Figure 7-3 Mean %Recovery ( $\pm 1$  s.d.) for cotinine (COT) from dichloromethane (DCM) and methanol (MeOH) after evaporation and reconstitution 200  $\mu$ l methanol with 0.1% formic acid**

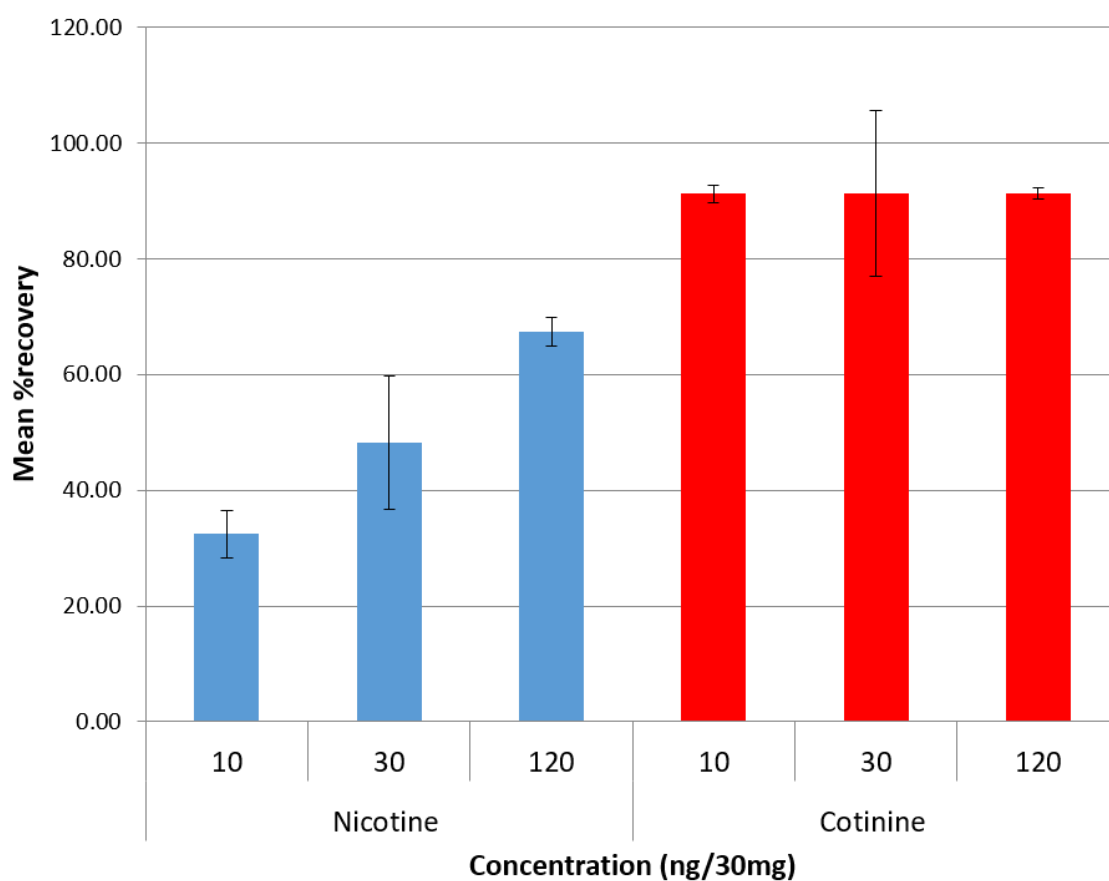
Since NIC is a basic drug, it is highly possible that it interacts with the surface of the vial walls and there is a need for using silanised vials. The same recovery study above was repeated using silanised vials for recovery from methanol. Better results were obtained and %recovery increased to about 60% for nicotine. However, it was observed that the work on silanised vials was not reproducible and high variations were noticed between concentrations and between replicates. Both evaporation of volatile nicotine and possible interaction with the vial walls makes it difficult to do either LLE or SPE as both methods involve an evaporation step.



**Figure 7-4 Comparison of mean %Recovery ( $\pm$  1 s.d.) of nicotine (NIC) and cotinine (COT) in silanised and non-silanised vials**

#### 7.8.1.2 Filtration

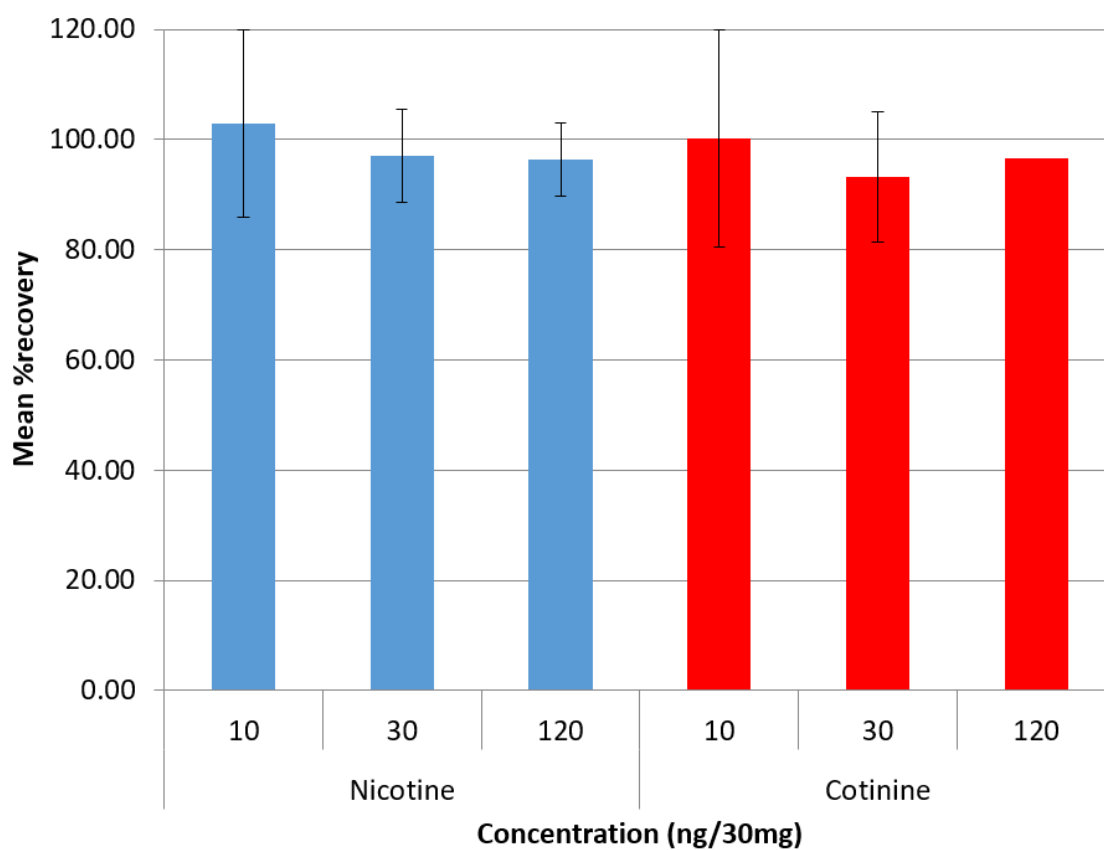
Recovery of cotinine was found to be very good ranging from 85 to 95%. Recovery of nicotine was found to be poor with low concentrations (approximately 30%) and, unexpectedly, the recovery improved with increasing concentrations (about 65% for the highest concentration) as shown in Figure 5-7. One possible explanation for this could be that nicotine may have a stronger affinity to the inner filter particles and becomes saturated quickly resulting in a better recovery with higher concentrations. This means this filter is not suitable for fur analysis as low concentrations of nicotine and cotinine are more likely to be detected from ETS exposure.



**Figure 7-5 Mean %recovery ( $\pm 1$  s.d.) from PES filter for nicotine (NIC) and cotinine (COT)**

### 7.8.1.3 Centrifugation

Recovery of nicotine and cotinine was found to be very good ranging from 95 to 110% as shown in Figure 7-6.



**Figure 7-6 Mean %recovery (+/- 1 s.d.) of nicotine (NIC) and cotinine (COT) after centrifugation**

#### 7.8.1.4 Summary of recovery studies for clean-up methods

The results of extraction recovery of different clean-up methods are summarised in Figure 7-7.

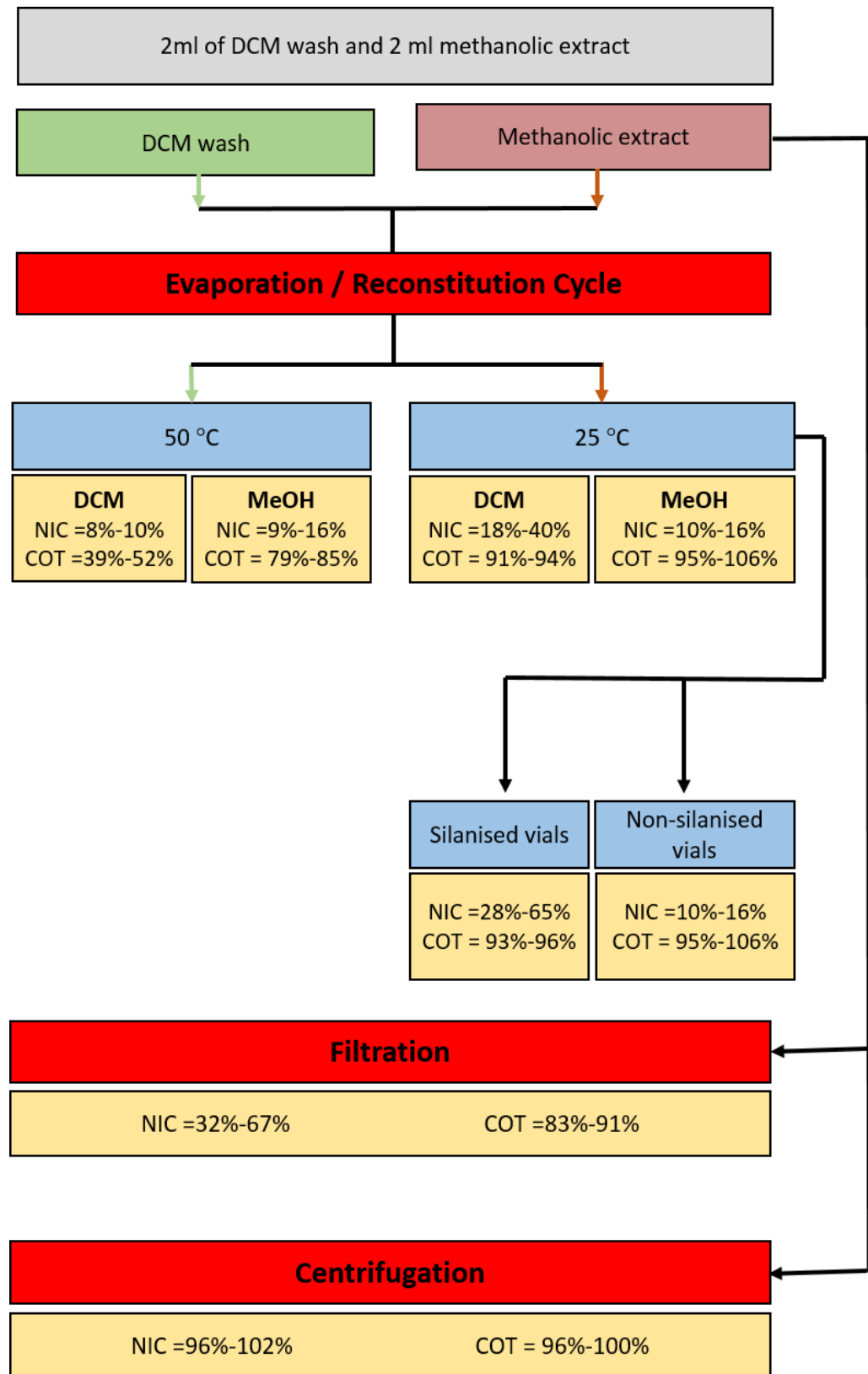


Figure 7-7 Summary of recovery study results of clean-up methods



## 7.8.2 Injection Volume

Using the simple volume of a cylinder equation, the volume of the column with dimensions mentioned previously in the materials section is 2.5 mL. However, 2.5 mL does not represent the actual volume of the column as it is packed with particles, which occupy 30-40% of the column volume. Thus, 60% of 2.5 mL is 1.5 mL, and 1% of that is 15  $\mu$ L. Increasing the injection volume without affecting the quality of the chromatogram is desirable, as this will improve the sensitivity. Hence, three different injection volumes (15, 20 and 50  $\mu$ L) were assessed for the sensitivity of chromatographic behaviour. 50  $\mu$ L injection results in very poor chromatography, 20  $\mu$ L gave satisfactory sensitivity without affecting the peak shape. Therefore, a 20  $\mu$ L injection volume was selected prior to validation.

## 7.8.3 Method validation results

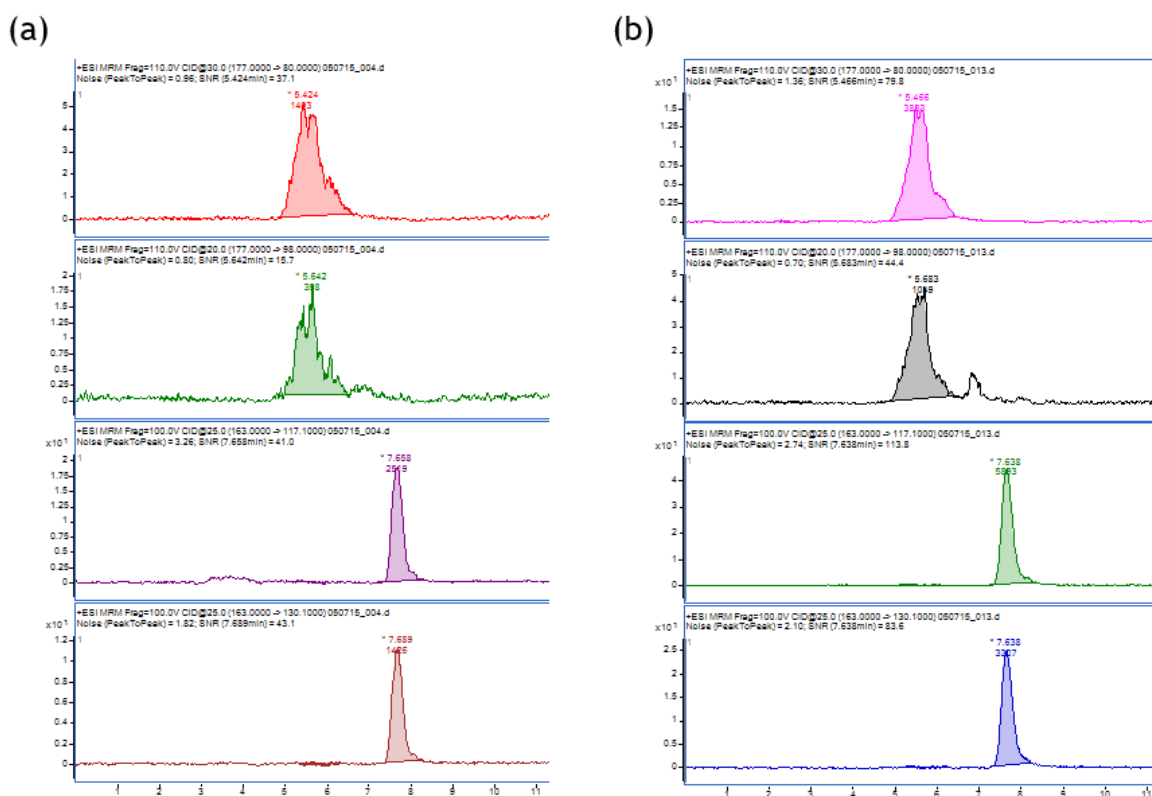
### 7.8.3.1 Limit of detection (LOD) and Lower Limit of quantitation (LLOQ)

The LOD and LLOQ for nicotine and cotinine are summarised in the Table 7-6.

**Table 7-6 LOD and LLOQ for nicotine and cotinine**

	Nicotine (ng/mg)	Cotinine (ng/mg)
LOD	0.01	0.05
LLOQ	0.05	0.1

A cut-off of 0.045 ng/mg was used for NIC. This concentration was chosen as it was three times the signal observed in the pooled blank sample made from 21 dogs who were reported to live in a nicotine-free environment. 0.1 ng/mg was chosen as the cut-off for COT due to poor chromatography. Figure 7-8 shows chromatograms of NIC and COT at 0.05 and 0.1 ng/mg.

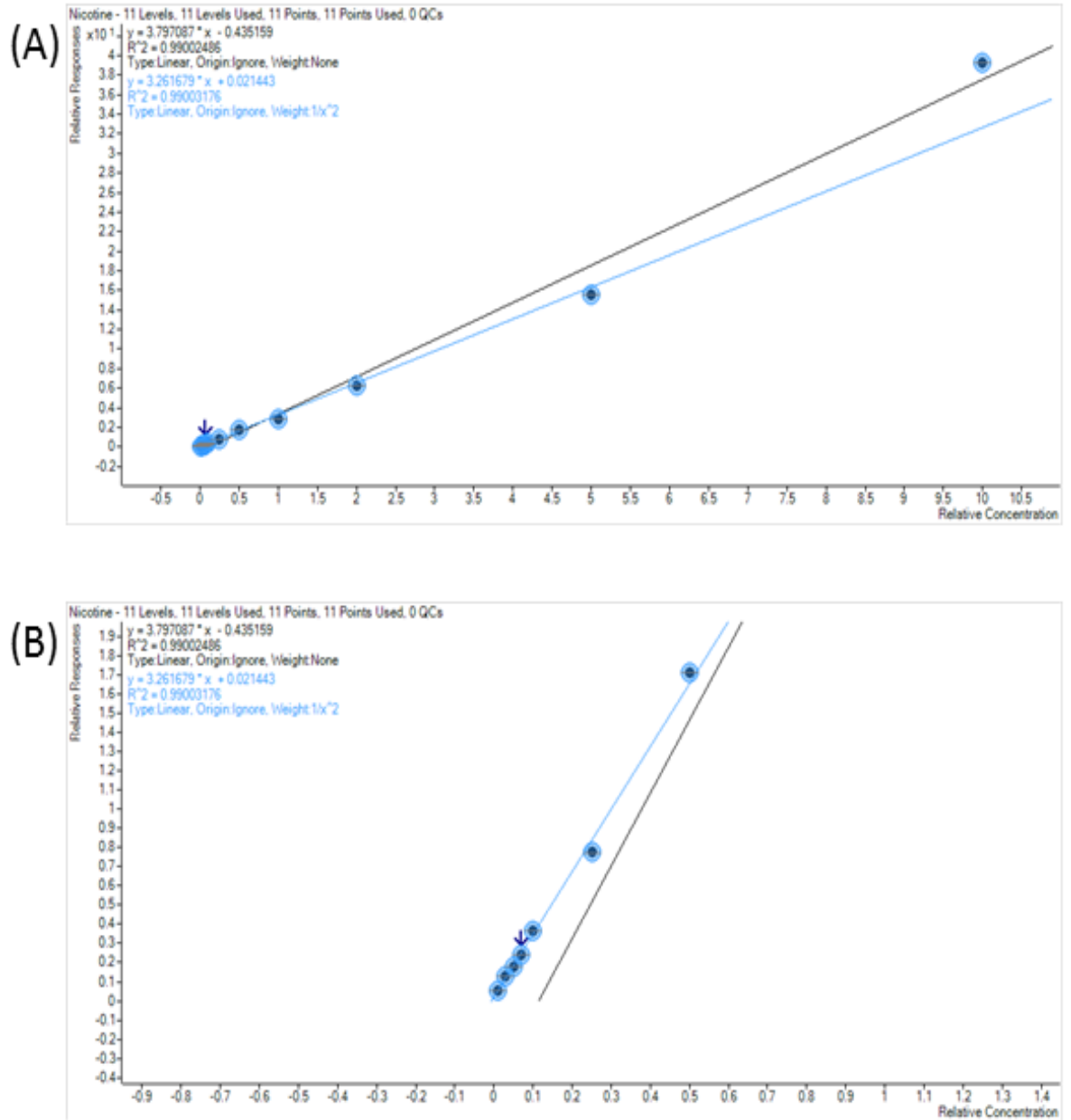


**Figure 7-8** Extracted chromatograms for nicotine (NIC) and cotinine (COT) transitions in standards containing (a) 0.05 ng/mg compared to (b) 0.1 ng/mg.

### 7.8.3.2 Calibration Model

The calibration curves were generated by plotting the peak area ratio versus the spiked analyte concentrations using the linear regression model on Agilent MassHunter Workstation-Quantitative software. Most LC calibration curves that span several orders of magnitude, such as our calibration model, show an increasing error with increasing concentration. For both analytes nicotine and cotinine, an excessive error was noticed at low quality control levels during validation. The presence of a heteroscedastic error in the calibration curve was investigated and confirmed using the *Gu et al* method (317). This error makes the data point at the high end of the calibration curve dominate the calculation of the linear regression and often results in an excessive error at the bottom of the curve. Using the linearity indicators approach,  $1/x^2$  was selected as the weighting factor (WF). Figure 7-9 B shows a clear example of how the curve is dominated by the high end point and resulting in inaccurate calculations at the low end. However, good accuracy can sometimes also be achieved in cases when unweighted curve was “luckily” overlaps with or is very close to the corresponding STD curve generated with the correct weighting factor as shown in Figure 7-10 B. R<sup>2</sup> values

were found to be better than 0.99 after  $1/x^2$  weighted regression over five replicates for nicotine and cotinine (see Figure 7-9 A and Figure 7-10 A). More details on the determination of heteroscedastic error and selection of the appropriate weighting factor are given in Appendix X.



**Figure 7-9 Nicotine calibration curve. (A) The curve ranging from 0.05 to 10 ng/mg, (B) The curve at the low concentration end ranging from 0.05 to 0.5 ng/mg.**

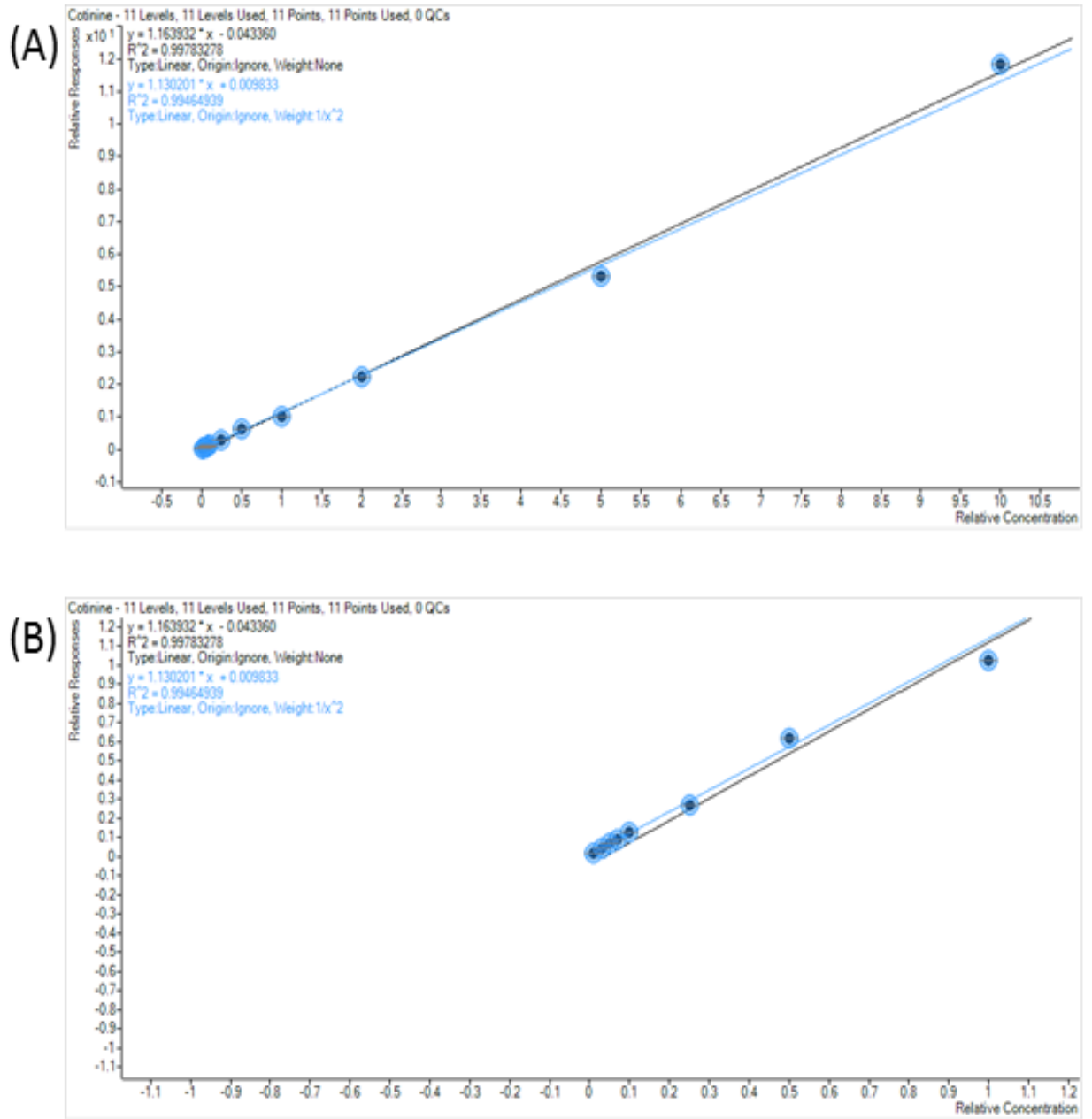


Figure 7-10 Cotinine calibration curve. (A) The curve ranging from 0.05 to 10 ng/mg, (B) The curve at the low concentration end ranging from 0.05 to 1 ng/mg.

### 7.8.3.3 Accuracy and precision

Accuracy and precision data for the method are summarised below in Table 7-7 and Table 7-8.

**Table 7-7 Nicotine quantitative results (ng/mg) of bias and precision runs. Run2 values considered an outlier and removed.**

<b>Low (0.05 ng/mg)</b>	<b>run1</b>	<b>run2</b>	<b>run3</b>	<b>run4</b>	<b>run5</b>
repl.1	0.05	0.02	0.07	0.06	0.06
repl.2	0.06	0.03	0.07	0.07	0.05
repl.3	0.06	0.02	0.07	0.07	0.06
<b>Medium (1ng/mg)</b>	<b>run1</b>	<b>run2</b>	<b>run3</b>	<b>run4</b>	<b>run5</b>
repl.1	1.11	0.87	0.86	1.19	1.07
repl.2	0.92	0.90	1.05	1.20	1.11
repl.3	0.87	0.92	1.14	1.04	0.86
<b>High (5 ng/mg)</b>	<b>run1</b>	<b>run2</b>	<b>run3</b>	<b>run4</b>	<b>run5</b>
repl.1	5.60	5.49	4.46	5.25	4.64
repl.2	5.07	5.21	4.29	6.36	4.81
repl.3	5.08	5.24	4.72	5.73	4.73

T-test was performed to compare the mean from run2 for nicotine with the other 4 runs. It was found that the set of data from run2 (highlighted in red) was significantly different from the other 4 runs. Therefore, run2 was considered as an outlier and excluded from the mean, accuracy and precision calculations. The poor quality of the blank fur is believed to be the reason for the noticed inaccuracy of the quantitation results for the lowest QC material, and therefore, the validation result was considered acceptable at this level.

Table 7-8 Cotinine quantitative results (ng/mg) of bias and precision runs

Low (0.05 ng/mg)	run1	run2	run3	run4	run5
repl.1	0.05	0.04	0.05	0.05	0.05
repl.2	0.05	0.04	0.05	0.05	0.04
repl.3	0.05	0.04	0.06	0.05	0.05
Medium (1ng/mg)	run1	run2	run3	run4	run5
repl.1	1.00	1.04	0.85	0.98	0.99
repl.2	0.98	1.04	0.98	1.01	0.91
repl.3	0.88	1.00	1.00	0.92	0.90
High (5 ng/mg)	run1	run2	run3	run4	run5
repl.1	4.86	5.01	4.70	4.72	4.34
repl.2	4.41	4.95	4.76	5.37	4.86
repl.3	4.53	5.25	4.68	5.37	4.37

Table 7-9 Summary of mean, precision and accuracy results. values in brackets are after removing outlier run2.

		Low (0.05 ng/mg)	Med (1ng/mg)	High (5 ng/mg)
NIC	Mean (ng/mg)	0.05 (0.62)	1.01	5.11
	Accuracy	8.23 (23.65)	0.66	2.26
	Within-Run CV (%)	1.22 (1.14)	11.21	13.80
	Between-Run CV (%)	59.07 (20.9)	22.19	19.31
COT	Mean (ng/mg)	0.05	0.97	4.81
	Accuracy	-3.43	-3.44	-3.74
	Within-Run CV (%)	1.17	5.70	11.36
	Between-Run CV (%)	14.38	10.50	12.57

#### 7.8.3.4 Matrix Effects

As shown in Figure 7-11, the matrix effect results (n=3) showed an enhancement for nicotine and its deuterated ISTD and suppression for cotinine and its deuterated ISTD. The percentage of matrix effect is averaged to be within  $\pm 25\%$  for both drugs which is, according to SWGTOX guidelines, an acceptable matrix effect.

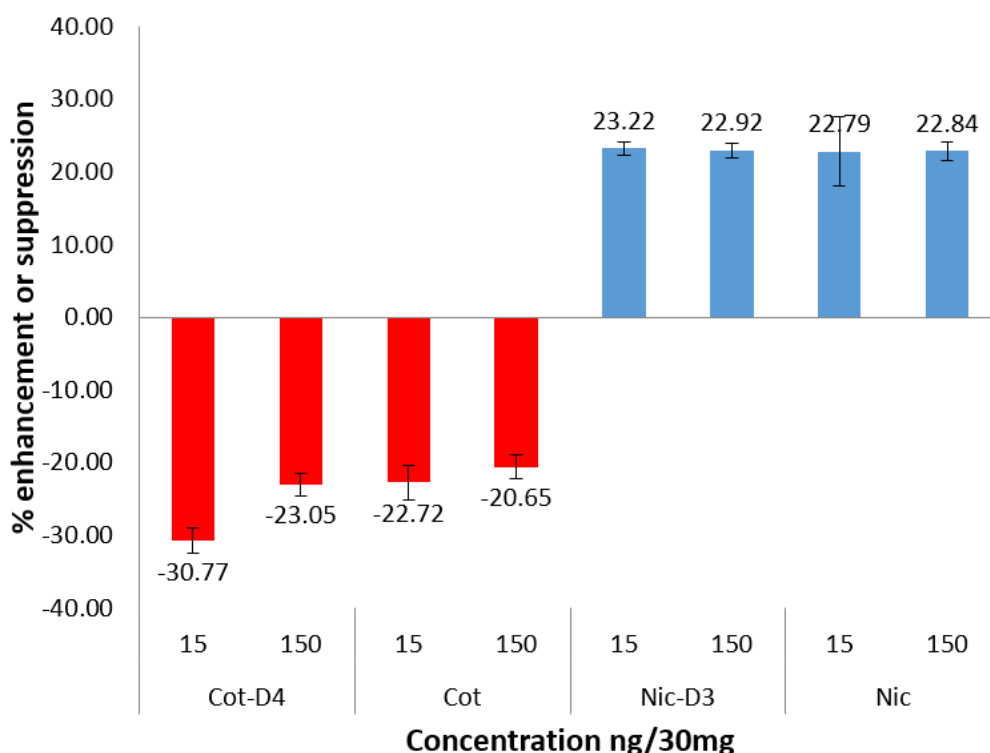


Figure 7-11 Mean matrix effect (n=3) (+/- 1 s.d.)

#### 7.8.3.5 Carryover

No signal was observed in the blank extraction solvent at the retention time for nicotine or cotinine. Carryover was therefore not deemed to be a problem.

### 7.9 Application to case samples

The developed method was applied to 66 fur samples which were collected by a postgraduate doctoral student enrolled at the University of Glasgow School of Veterinary Science. The samples were stored at room temperature away from light



until analysis by LC/MS-MS. These fur samples were subdivided into two groups: the first group contained samples with a sufficient quantity that allowed for multiple analyses. As samples were collected for the same dogs at two different time points, 15 pairs were found to have a sufficient quantity of fur. This group of samples were used to assess the optimised methanolic extraction. The second group consisted of all collected fur samples and were used to quantify the total nicotine and cotinine. The results of the analyses of fur samples will be reported in the next chapter. Figure 7-12 summarises the work reported in this chapter.

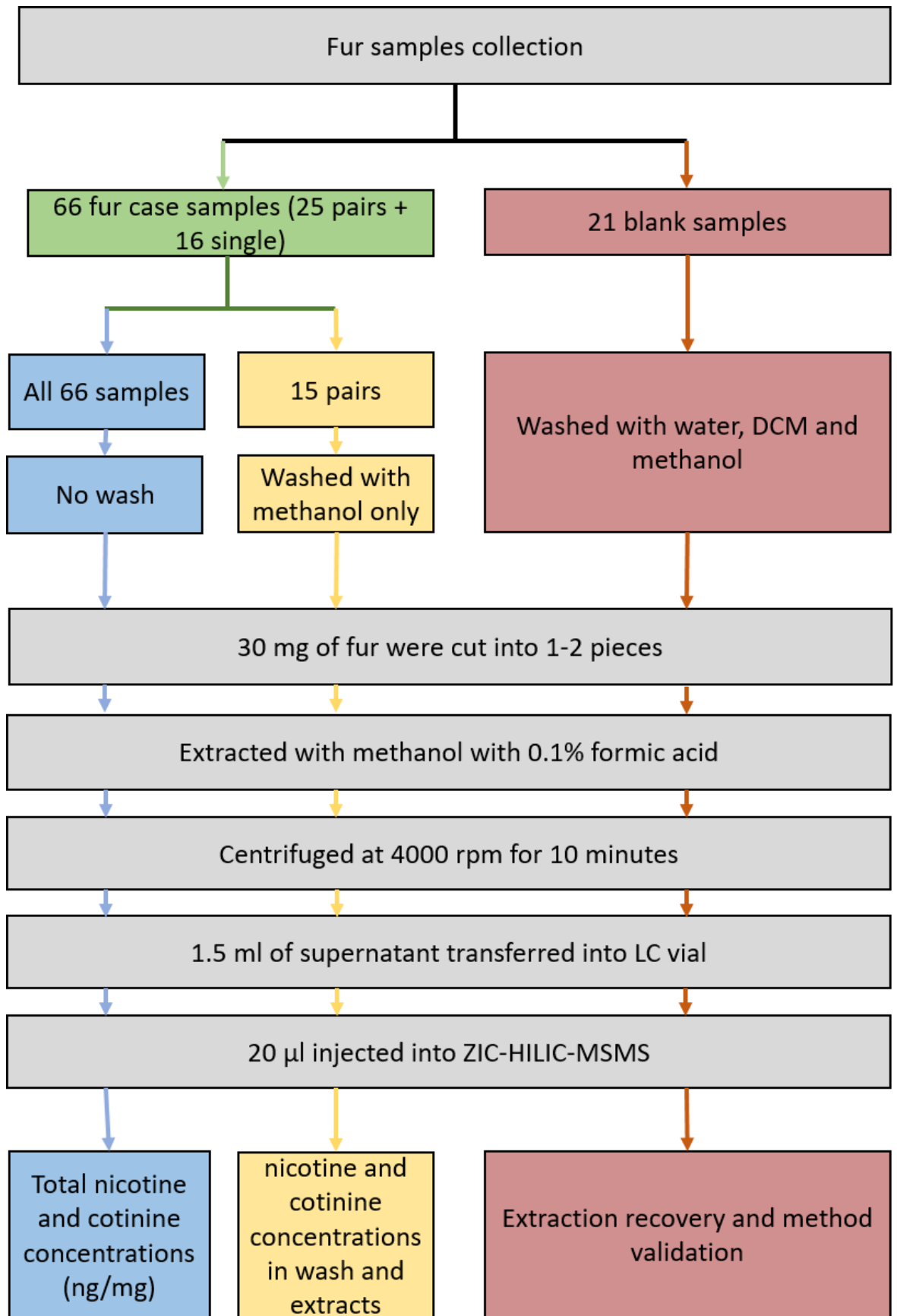


Figure 7-12 Project summary

## 7.10 Conclusion

LC-MS/MS has become a very important tool for forensic toxicology investigations. Nicotine and its primary metabolite, cotinine in hair have proven to be sensitive and specific biomarkers for ETS exposure investigations. Low cost, fast extraction procedures or new materials for extraction and analyte separation is now a trend in the analytical laboratory. A direct injection procedure for fur methanolic extracts with simple sample pre-treatment was employed in this method. Nicotine loss during sample preparation, either by evaporation or interaction with vial walls, still requires further investigation. Matrix effects are a major drawback in modern analytical applications using LC-MS/MS; a proper dilution might be needed to reduce the matrix effects. Despite the limited applications for ZIC-HILIC columns in forensic toxicology, it has promising separation capabilities for the polar compounds. The column compatibility with a wide range of solvents made it possible to inject MS friendly high organic content straight into the mobile phase. This is believed to enhance the response of ESI-MS/MS. Highly organic mobile phases are more volatile which increases ESI-MS sensitivity through improving the efficiency of the formation of gas phase ions. Direct injection of high organic content solutions has also shortened the analysis time by eliminating the need for the evaporation step and sample reconstitution with a suitable solvent prior to injection into the LC-MS.

## **Chapter 8 Application of fur NIC and COT method to dogs exposed to environmental smoke exposure**

### **8.1 Introduction**

The method described in Chapter 8 was applied to fur specimens received by the laboratory from the University of Glasgow School of Veterinary Science for total nicotine and cotinine quantitation. The study was undertaken to first establish whether dogs are exposed to significant, detectable amounts of ETS in the home, secondly, to evaluate the changes in ETS exposure in the time between two sample collection times and, thirdly, to correlate the measured concentrations with owner-reported exposure to ETS. In addition, methanolic wash efficiency and optimisation of the extraction incubation period were investigated.

### **8.2 Aims**

The aims of the study were to:

- Establish the validity of nicotine and cotinine to measure ETS exposure.
- Compare the concentrations of nicotine and cotinine in the positive fur samples exposed to different levels of ETS.
- Compare the concentrations of nicotine and cotinine with concentrations reported previously from human and animal hair samples exposed to ETS.
- Establish whether companion animals fur samples could be used to reflect the long-term ETS exposure in homes.
- Compare the sensitivity, specificity and likelihood ratio for both nicotine and cotinine assays for monitoring ETS.
- Determine if a nicotine/cotinine ratio can be established to distinguish intensity of exposure to ETS.

### 8.3 Fur sample collection and labelling

Fur collection from dogs was carried out by a postgraduate doctoral student enrolled at the University of Glasgow School of Veterinary Science by clipping mainly the neck region with the owner's consent. To comply with ethical approval, fur that was removed from the site of blood collection was used for analysis, therefore, two different collection sites were reported for some dogs (highlighted in blue in Table 8-1). Owners were asked to complete a questionnaire briefly outlining the age, breed and sex of the dog and estimating the amount of ETS to which each dog was exposed. Non-exposed were defined as dogs with a non-smoker owner or family member, who declared that their dogs had no unusual environmental exposure to tobacco smoke. Occasionally exposed were defined as those dogs living with an owner or family member who smokes only outdoor. Passive smokers were defined as those dogs living with a smoker owner or family member, who smoked in the presence of the dog. The collected fur was stored in sealed collection envelopes and stored at room temperature to ensure that no further ETS exposure occurred after hair collection. At the time of analysis, the fur samples were transferred from the envelope with tweezers to Chromacol Environmental Sampling Vials. Scissors used for cutting and tweezers were cleaned with methanol before use and in between samples. Fur samples were subsequently prepared and extracted following the methods and procedure explained earlier in chapter 8. Each fur sample was allocated a sequential laboratory number starting from 1. If two samples were available for the same dog, the first collected sample was labelled as 1-A and the second as 1-B. Samples from 1 to 25 were all from dogs where two samples were collected. Samples 26 to 41 were from dogs that did not return for a second sample and were all labelled with A. Methanolic washes were given a laboratory number for their sample ID with an added (M) at the end. As an example, washes and extract from sample number 1 were labelled as follow; 1-A, 1-B, 1-A-M and 1-B-M.

### 8.4 Fur samples submitted for analysis

Between March 2013 and February 2015, a total of 66 samples were collected from 41 dogs. 25 dogs returned for the follow-up appointment and a second sample collected. The mean and median for the net days between the two sample collection dates were  $m=281.6$  days and  $M=273.5$  days. All samples were stored in

clean and dark place at room temperature prior to analysis. Demographic information for all samples is summarized in Table 8-1.

**Table 8-1 Fur sample information**

<b>Dog No.</b>	<b>Time point</b>	<b>Fur colour</b>	<b>Smoke exposure status</b>
<b>1</b>	First sample	Black	Smoking occurs indoor
	Second Sample	Black	Smoking occurs indoor
<b>2</b>	First sample	White	No Exposure
	Second Sample	White	No Exposure
<b>3</b>	First sample	Black	No Exposure
	Second Sample	Black	No Exposure
<b>4</b>	First sample	Light Brown	Smoking occurs indoor
	Second Sample*	Black	Smoking occurs indoor
<b>5</b>	First sample	Black	Smoking occurs indoor
	Second Sample	Black	Smoking occurs indoor
<b>6</b>	First sample	White	No Exposure
	Second Sample	White	No Exposure
<b>7</b>	First sample	White	Smoking occurs only outdoor
	Second Sample	White	No Exposure
<b>8</b>	First sample	Light Brown	No Exposure
	Second Sample	Light Brown	No Exposure
<b>9</b>	First sample	Light Brown	Smoking occurs indoor
	Second Sample	Grey	Smoking occurs indoor
<b>10</b>	First sample	White	No Exposure
	Second Sample	White	No Exposure
<b>11</b>	First sample	Grey	Smoking occurs indoor
	Second Sample	White	No Exposure
<b>12</b>	First sample	Black	No Exposure
	Second Sample	Brown	No Exposure
<b>13</b>	First sample	Brown	Smoking occurs indoor
	Second Sample	Brown	Smoking occurs only outdoor
<b>14</b>	First sample	White	No Exposure
	Second Sample	White	No Exposure
<b>15</b>	First sample	Brindle	No Exposure
	Second Sample	Brindle	No Exposure
<b>16</b>	First sample	Black	Smoking occurs indoor
	Second Sample	White	No Exposure
<b>17</b>	First sample	White	No Exposure
	Second Sample	Grey	No Exposure
<b>18</b>	First sample	Light Brown	No Exposure
	Second Sample	Light Brown	No Exposure
<b>19</b>	First sample	Grey	No Exposure
	Second Sample	Grey	No Exposure
<b>20</b>	First sample	Brindle	Smoking occurs indoor

	Second Sample	Light Brown	No Exposure
21	First sample	White	No Exposure
	Second Sample	White	No Exposure
22	First sample	Black	Smoking occurs only outdoor
	Second Sample	Black	Smoking occurs only outdoor
23	First sample	Black	No Exposure
	Second Sample	Black	No Exposure
24	First sample	Black	No Exposure
	Second Sample	Black	No Exposure
25	First sample	Grey	Smoking occurs only outdoor
	Second Sample	Grey	Smoking occurs only outdoor
26	First sample	Grey	No Exposure
27	First sample	Light Brown	Smoking occurs indoor
28	First sample	Light Brown	No Exposure
29	First sample	Black	Smoking occurs indoor
30	First sample	Brown	Smoking occurs indoor
31	First sample	Black	Smoking occurs only outdoor
32	First sample	Black	No Exposure
33	First sample	White	No Exposure
34	First sample	White	Smoking occurs only outdoor
35	First sample	White	No Exposure
36	First sample	Black	Smoking occurs indoor
37	First sample	Dark Brown	No Exposure
38	First sample	Light Brown	Smoking occurs indoor
39	First sample	Black	No Exposure
40	First sample	Light Brown	Smoking occurs indoor
41	First sample	Brown	No Exposure

Sample numbers highlighted in grey were selected to carry out the evaluation of methanol solvent for washing extraction. Samples highlighted in light blue (fur colour) were collected from different anatomical regions.

## 8.5 Analysis

### 8.5.1 Fur analysis

Analysis of fur samples was carried out using the analytical method described previously in chapter 8. All 66 submitted samples were analysed for total nicotine and total cotinine without a prior washing step at the collector's request. For evaluation of methanol as a washing and extraction agent, 30 samples, out of the 66 fur samples, only were selected and retested. Fur samples from dogs that did not return for the second sample (n=16) were excluded. From the remaining samples (n=50), only pairs where there was sufficient quantity were selected for analysis (n=15 pairs). Fur samples were washed with methanol for 15 minutes with

sonication. Fur was then extracted with methanol containing 0.1% formic acid for two hours. 200 µl aliquots were collected from each sample at different times (15, 30, 60 and 120 minutes) during sonication and transferred to LC vials for injection. The methanol wash was centrifuged to remove particulates before injection. The extracted aliquots were difficult to centrifuge, and therefore, they were left on the bench for 1-2 hours to allow particulates to precipitate before injection and no further clean-up was conducted.

### **8.5.2 Statistical Analysis**

Statistical analysis was carried out using Microsoft Excel® 2016 and Statistical Package for the Social Sciences (SPSS) software for Microsoft Windows version 22.0 (IBM Corporation, Armonk, New York, US). In sections 9.8.1 to 9.8.5, statistical analysis was carried out on the concentrations of nicotine and cotinine detected in dogs exposed to different levels of ETS. The distribution of the concentrations of each analyte was assessed using the Shapiro-Wilk test and found not to follow a normal distribution. Thus, non-parametric tests were used to test for significant differences between the two time points and relationships between the questionnaire-estimated levels of exposure and observed fur colour for the distribution of nicotine and cotinine concentrations. The Kruskal-Wallis test, the nonparametric analogue for ANOVA, was used to compare the means of more than two groups (e.g. concentration of nicotine and cotinine in the three levels of exposure to ETS or different fur colour). The Mann-Whitney test was used for assessing the mean difference of concentrations at the two time points. A p value < 0.05 was considered significant. Spearman's Rank Correlation Test (2-tailed) was used to assess the strength and direction of the association between the two continuous variables, nicotine and cotinine concentrations

### **8.5.3 Sensitivity, specificity, predictive value and likelihood ratio calculations**

This method is simple, fast and does not require laborious and extensive sample preparation. Therefore, it can be used to qualitatively screen a large number of samples for the presence of nicotine and cotinine. Sensitivity, specificity, predictive value and likelihood ratio are the main performance characteristics of a binary classification test. Sensitivity measures the percentage of positives that



are truly identified as such (e.g., the percentage of smoke-exposed dogs who are truly identified as positive for nicotine or cotinine). Specificity measures the proportion of negatives that are correctly identified as such (e.g., the percentage of non-exposed dogs who are correctly identified as negative for nicotine and cotinine). The positive and negative predictive values (PPV and NPV respectively) are the proportions of positive and negative results in diagnostic tests that are true positive and true negative results. The likelihood ratio is an additional measure that uses both the sensitivity and specificity of the test to determine whether a test result usefully changes the probability that an exposure to smoke exists. Two forms of the likelihood ratio occur, positive likelihood ratio for positive test results (LR+) and negative likelihood ratio (LR-) for negative test results.

The reference results of ETS exposure were based on the assumption that exposure status reported by owners in the questionnaire were accurate. Dogs living with a smoker were considered exposed while those living with non-smokers were classified as not exposed. For ETS exposure screening, a perfect biomarker would be described as 100% sensitive and 100% specific. Sensitivity, specificity and both versions of predictive value and likelihood ratio is calculated as shown in the following Equation 8-1 Equation 8-6.

**Equation 8-1 Sensitivity equation**

$$\text{sensitivity} = \frac{\text{number of true positives (TP)}}{\text{number of TP} + \text{number of false negatives (FN)}} * 100$$

**Equation 8-2 Specificity equation**

$$\text{specificity} = \frac{\text{number of true negatives (TN)}}{\text{number of TN} + \text{number of false positives (FP)}} * 100$$

**Equation 8-3 Positive predictive value**

$$PPV = \frac{TP}{TP + FP}$$

**Equation 8-4 Negative predictive value**

$$NPV = \frac{TN}{TN + FN}$$

**Equation 8-5 Positive likelihood ratio equation**

$$LR+ = \frac{sensitivity}{1 - specificity}$$

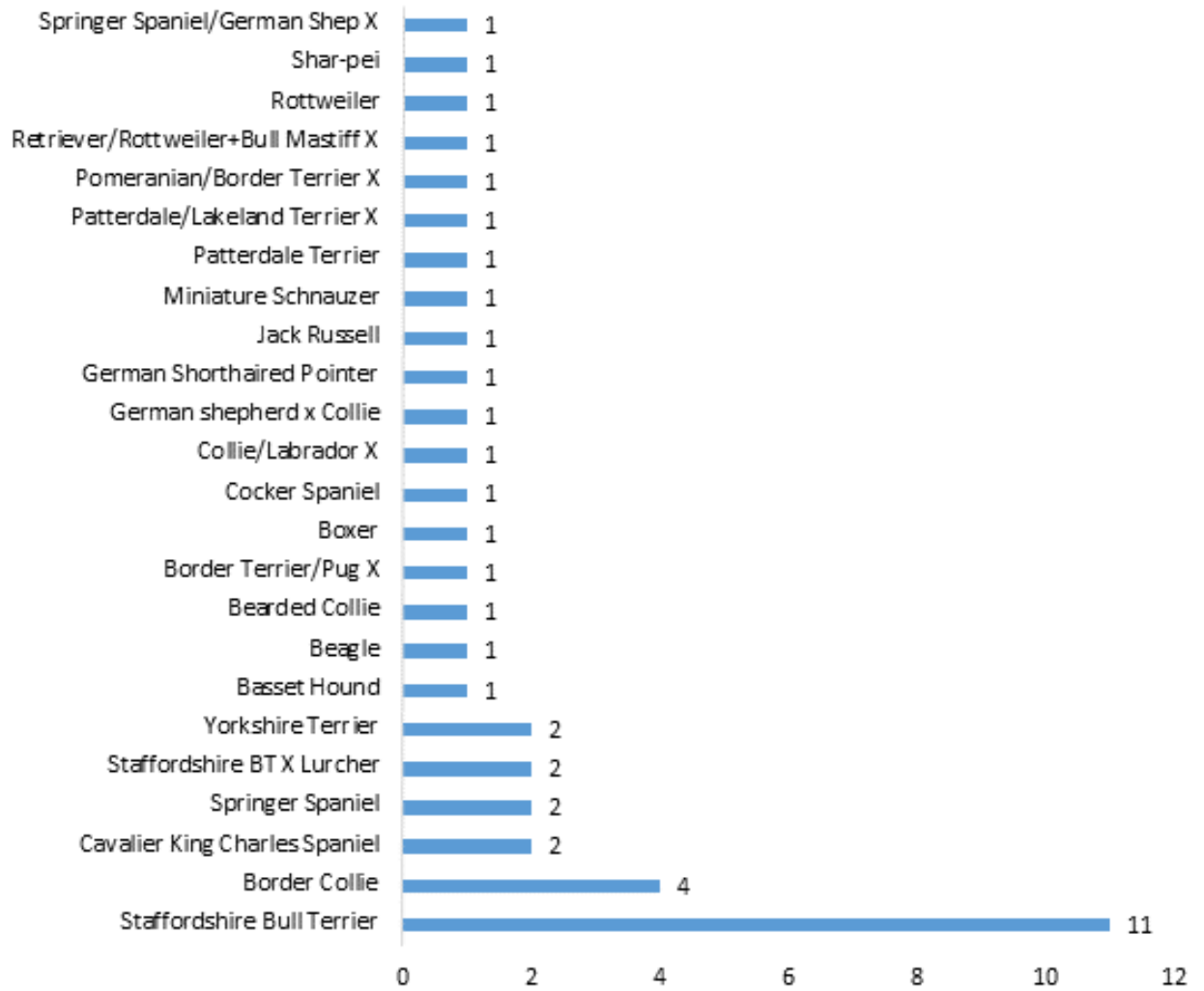
**Equation 8-6 Negative likelihood ratio equation**

$$LR- = \frac{1 - sensitivity}{specificity}$$

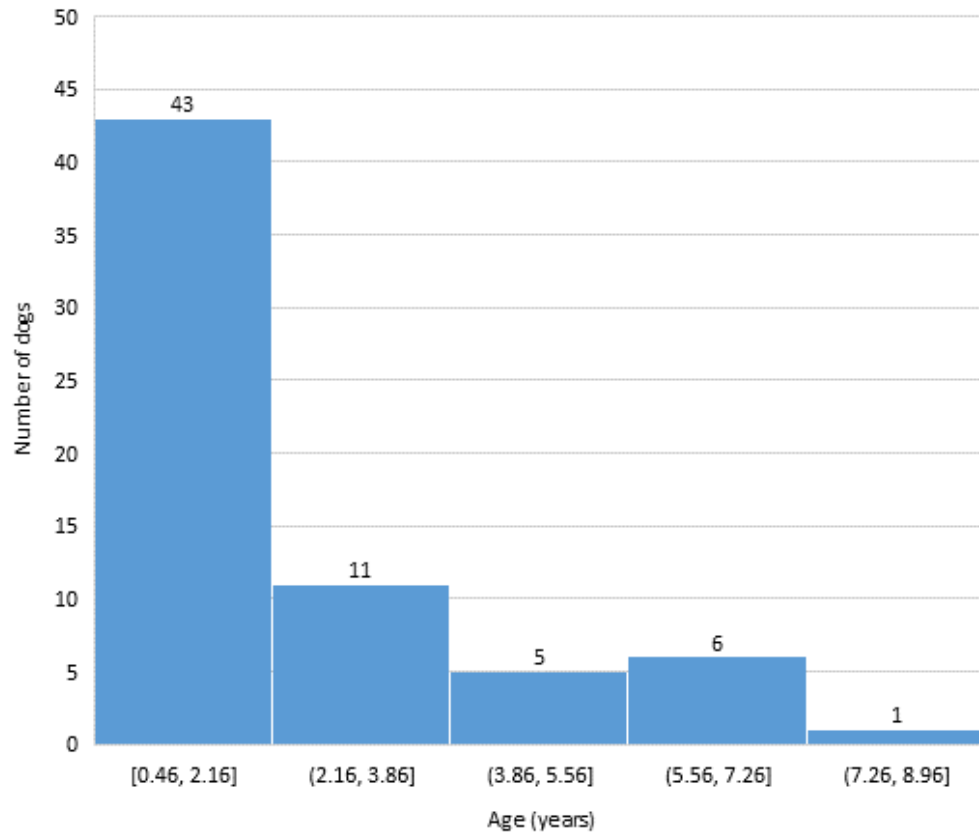
## 8.6 Results – demographic

### 8.6.1 Age, weight and breed

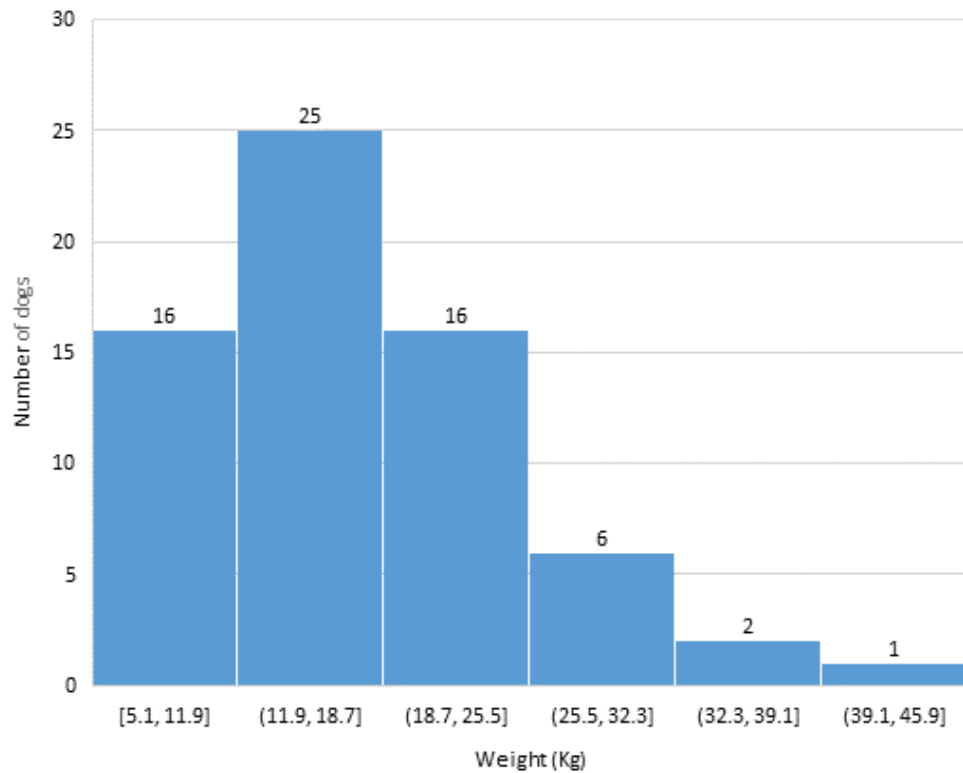
The 66 fur samples analysed for nicotine and cotinine were collected from 41 dogs from 24 different breeds. The Staffordshire Bull Terrier breed was predominant with more than 20% of dogs (Figure 8-1). The frequency of fur samples was spread across the age range 0.4 to 8.9 years old, with 65% of cases between the ages of six months to two years old (Figure 8-2). The weight of dogs involved in the study ranged from 5 kg to 45 kg (Figure 8-3).



**Figure 8-1 Number of fur samples included in analysis classified by dog breed**



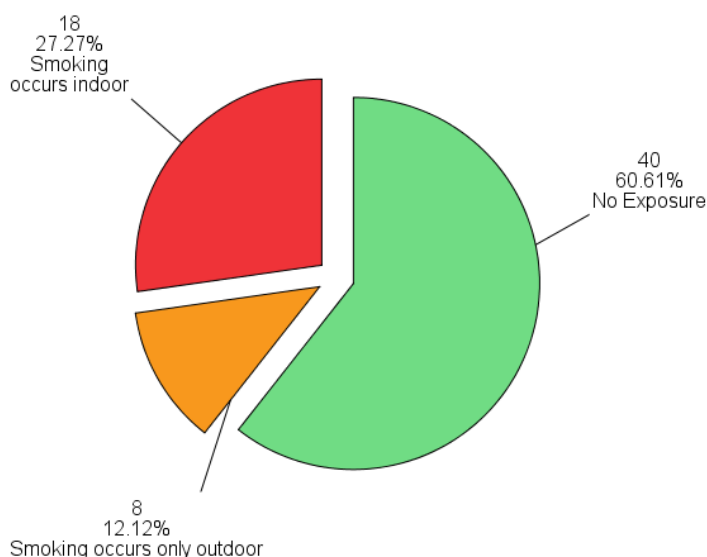
**Figure 8-2 Histogram showing number of fur samples included in analysis classified by age range**



**Figure 8-3 Histogram showing number of fur samples submitted for analysis classified by weight range**

### 8.6.2 Classification of fur samples

The fur samples were classified according to the degree of exposure reported by the owner in the questionnaire and based on their colour observed in the laboratory when the samples were received. Numbers and percentages of each class is shown in Figure 8-4 and Figure 8-5.



**Figure 8-4 Classification of fur samples based on the history of exposure to ETS**

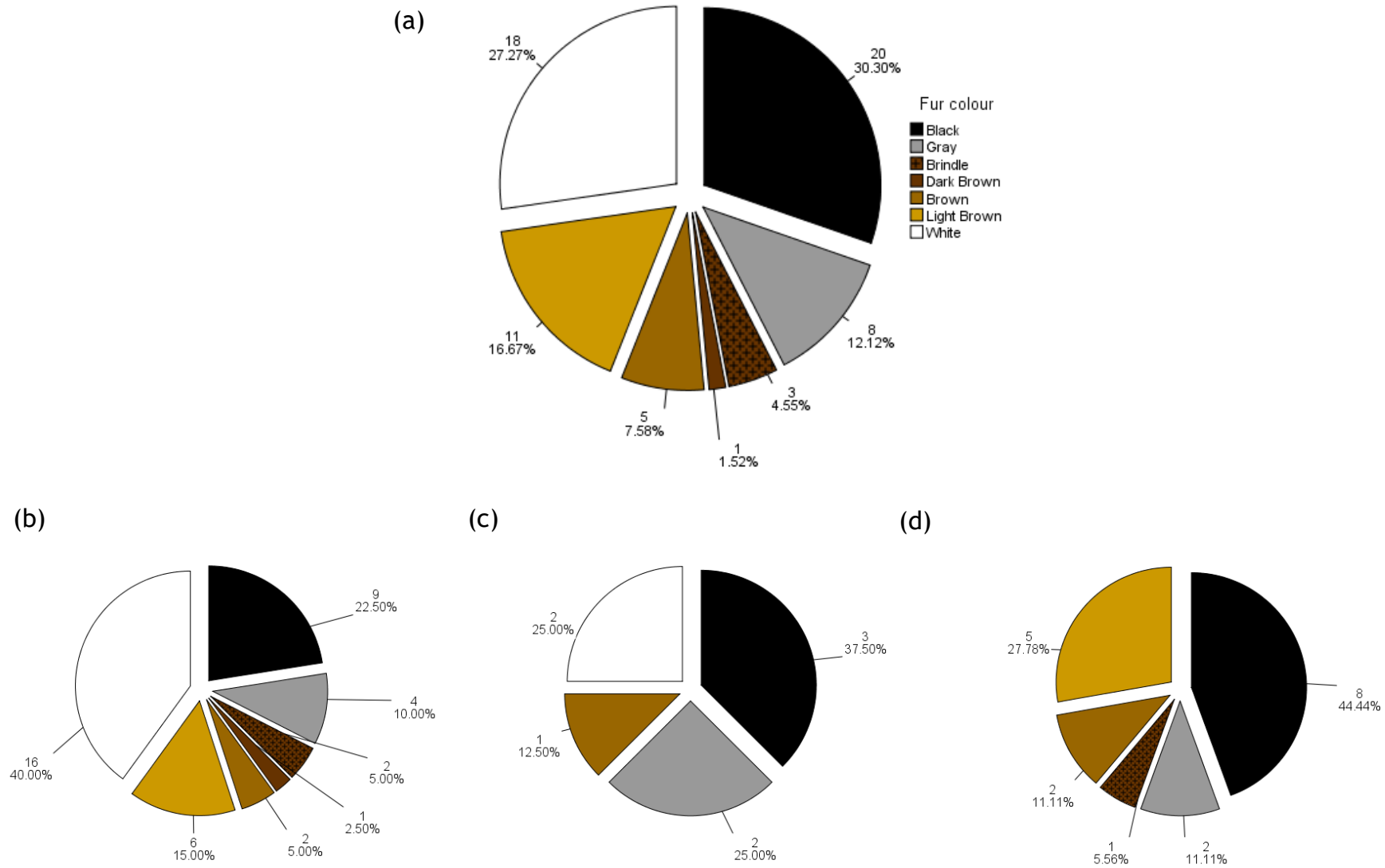
## 8.7 Results - quantitative and qualitative

### 8.7.1 Construction of calibration curves

Agilent MassHunter software was used to construct the calibration curves using the equation  $y = mx + c$  with  $1/x^2$  weighting factor and was used to calculate the % peak area ratios (% PAR) for each analyte (peak area for analyte/peak area for internal standard \* 100). The calibration lines for each analyte were constructed as ng/mg.

### 8.7.2 Summary of total nicotine and total cotinine concentrations

Concentrations of total nicotine and total cotinine in dogs' fur ranged from 0.05-13.7 ng/mg and 0.12-0.87 ng/mg, respectively. Table 8-2 summarises the total nicotine and total cotinine concentrations in all 66 fur samples.



**Figure 8-5 Classification of fur samples based on their colour. (a) in all exposure groups, (b) No exposure group only, (c) smoking occurs outdoor, (d) smoking occurs indoor.**

Table 8-2 Total nicotine and total cotinine concentrations in 66 fur samples

sample ID	Time point 1 (ng/mg)		Time point 2 (ng/mg)	
	Total NIC	Total COT	Total NIC	Total COT
1	4.05	0.21	12.58	0.48
2	0.07	ND	ND	ND
3	ND	ND	ND	ND
4	0.44	ND	0.33	ND
5	5.93	0.62	9.62	0.35
6	ND	ND	ND	ND
7	0.14	ND	0.36	ND
8	ND	ND	ND	ND
9	2.44	0.14	1.93	0.28
10	0.27	ND	0.10	ND
11	0.36	ND	0.20	0.21
12	ND	ND	ND	ND
13	0.45	0.15	1.31	0.71
14	ND	ND	ND	ND
15	ND	ND	ND	ND
16	1.08	ND	0.21	0.18
17	ND	ND	ND	ND
18	0.08	ND	0.15	ND
19	0.08	ND	ND	ND
20	0.81	0.19	0.09	ND
21	ND	ND	0.06	ND
22	0.08	ND	0.06	ND
23	0.05	ND	ND	ND
24	ND	ND	ND	ND
25	1.79	0.24	0.97	0.20
26	ND	ND		
27	0.65	0.13		
28	ND	ND		
29	0.58	0.12		
30	13.79	0.87		
31	0.16	ND		
32	0.06	ND		
33	ND	ND		
34	0.95	0.68		
35	ND	ND		
36	ND	ND		
37	0.47	ND		
38	0.47	0.14		
39	0.14	ND		
40	1.73	0.12		
41	0.19	ND		

ND stands for 'Not detected'

Figure 8-6 display the extracted chromatograms for a fur specimen (1-B) that was positive for NIC and COT at both time points and has the second highest NIC concentration.

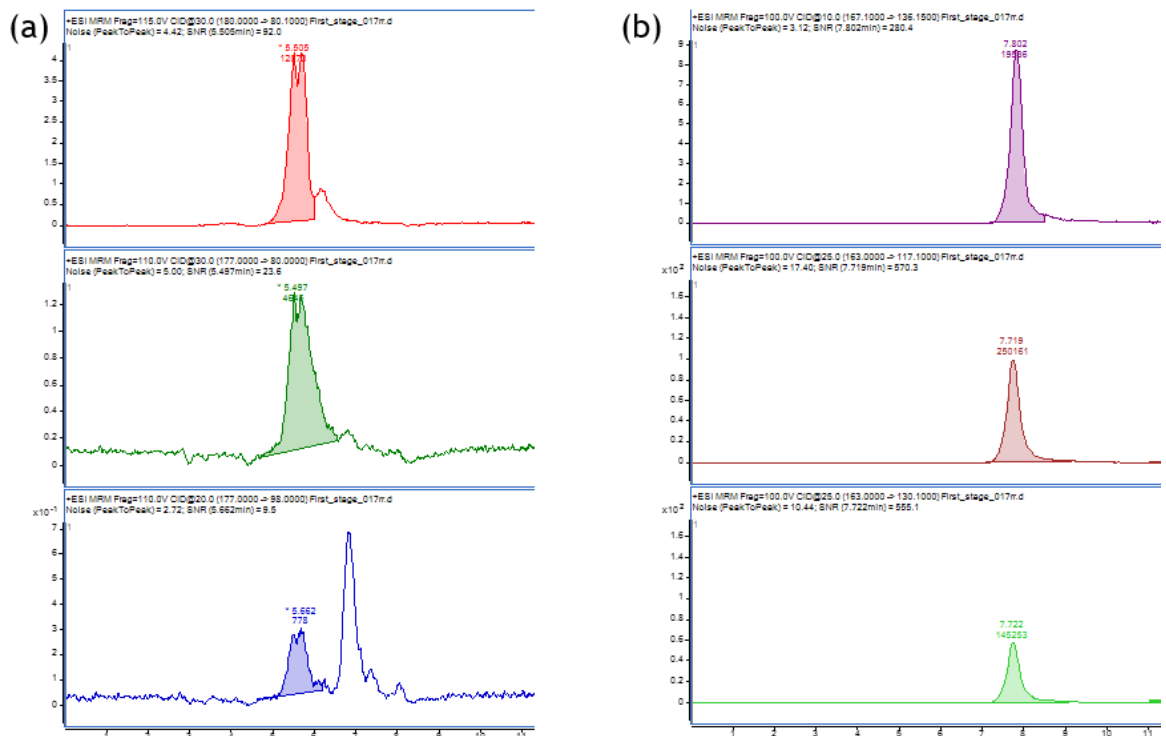


Figure 8-6 Extracted chromatograms for fur sample (1-B) containing (a) 0.48 ng/mg cotinine (COT), and (b) 12.58 ng/mg nicotine (NIC).



### 8.7.3 Summary of nicotine and cotinine concentrations in the methanolic wash and extract

The concentrations of nicotine and cotinine quantified in the methanol wash and in the extract are summarised in Table 8-3 and Table 8-4.

**Table 8-3 Summary of nicotine concentrations in the methanol washes and extracts**

Sample ID	Nicotine concentrations (ng/mg)				
	Methanol wash	Methanolic extract			
		15 min	30 min	60 min	120 min
1-A	2.14	0.72	1.17	2.04	2.42
1-B	5.92	2.05	2.81	3.72	4.16
3-A	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
3-B	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6-A	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
6-B	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
8-A	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
8-B	<LOQ	ND	ND	ND	ND
9-A	0.58	0.56	0.91	1.12	1.21
9-B	0.98	0.36	0.48	0.46	0.50
10-A	0.10	0.06	0.10	0.12	0.13
10-B	0.05	<LOQ	<LOQ	<LOQ	<LOQ
11-A	0.11	0.06	0.12	0.16	0.18
11-B	0.07	<LOQ	<LOQ	<LOQ	<LOQ
12-A	<LOQ	ND	ND	<LOQ	<LOQ
12-B	<LOQ	ND	ND	<LOQ	<LOQ
15-A	<LOQ	ND	<LOQ	<LOQ	<LOQ
15-B	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
16-A	0.50	0.20	0.23	0.26	0.29
16-B	0.09	<LOQ	0.05	0.05	0.05
20-A	0.55	<LOQ	<LOQ	<LOQ	<LOQ
20-B	0.05	0.12	0.16	0.20	0.25
21-A	<LOQ	ND	ND	ND	ND
21-B	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
22-A	0.05	<LOQ	<LOQ	<LOQ	<LOQ
22-B	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
24-A	<LOQ	ND	ND	ND	<LOQ
24-B	<LOQ	ND	ND	ND	ND
25-A	0.57	0.29	0.38	0.23	0.37
25-B	1.00	0.17	0.20	0.35	0.23

**Table 8-4 Summary of cotinine concentrations in the methanolic wash and extracts**

Cotinine concentrations (ng/mg)					
Sample ID	Methanol wash	Methanolic extract			
		15 min	30 min	60 min	15 min
1-A	0.15	<LOQ	<LOQ	0.13	0.18
1-B	0.31	0.12	0.17	0.16	0.15
3-A	ND	ND	ND	ND	ND
3-B	ND	ND	ND	ND	ND
6-A	ND	ND	ND	ND	ND
6-B	ND	ND	ND	ND	ND
8-A	ND	ND	ND	ND	ND
8-B	ND	ND	ND	ND	ND
9-A	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
9-B	0.17	<LOQ	<LOQ	<LOQ	<LOQ
10-A	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
10-B	ND	ND	ND	ND	ND
11-A	ND	ND	<LOQ	<LOQ	<LOQ
11-B	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
12-A	ND	ND	ND	ND	ND
12-A	ND	ND	ND	ND	ND
15-A	ND	ND	ND	ND	ND
15-B	ND	ND	ND	ND	ND
16-A	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
16-B	0.11	<LOQ	<LOQ	<LOQ	<LOQ
20-A	0.10	<LOQ	<LOQ	<LOQ	<LOQ
20-B	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
21-A	ND	ND	ND	ND	ND
21-B	ND	ND	ND	ND	ND
22-A	<LOQ	ND	ND	ND	ND
22-B	ND	ND	ND	ND	ND
24-A	ND	ND	ND	ND	ND
24-B	ND	ND	ND	ND	ND
25-A	0.17	<LOQ	<LOQ	<LOQ	<LOQ
25-B	0.19	<LOQ	<LOQ	<LOQ	<LOQ

### 8.7.4 Sensitivity, specificity, predictive value and likelihood ratio

The following three Table 8-5, Table 8-6 and Table 8-7 summarise the results of nicotine and cotinine against the reported exposure status in the questionnaire. Dogs in the second and third exposure groups with smoking owners were grouped together as 'exposed'.

**Table 8-5 Nicotine results for exposed and non-exposed dogs**

	Smoking status		Totals
	Not exposed	Exposed	
<b>Test positive</b>	16	25	41
<b>Test negative</b>	24	1	25
<b>Totals</b>	40	26	66

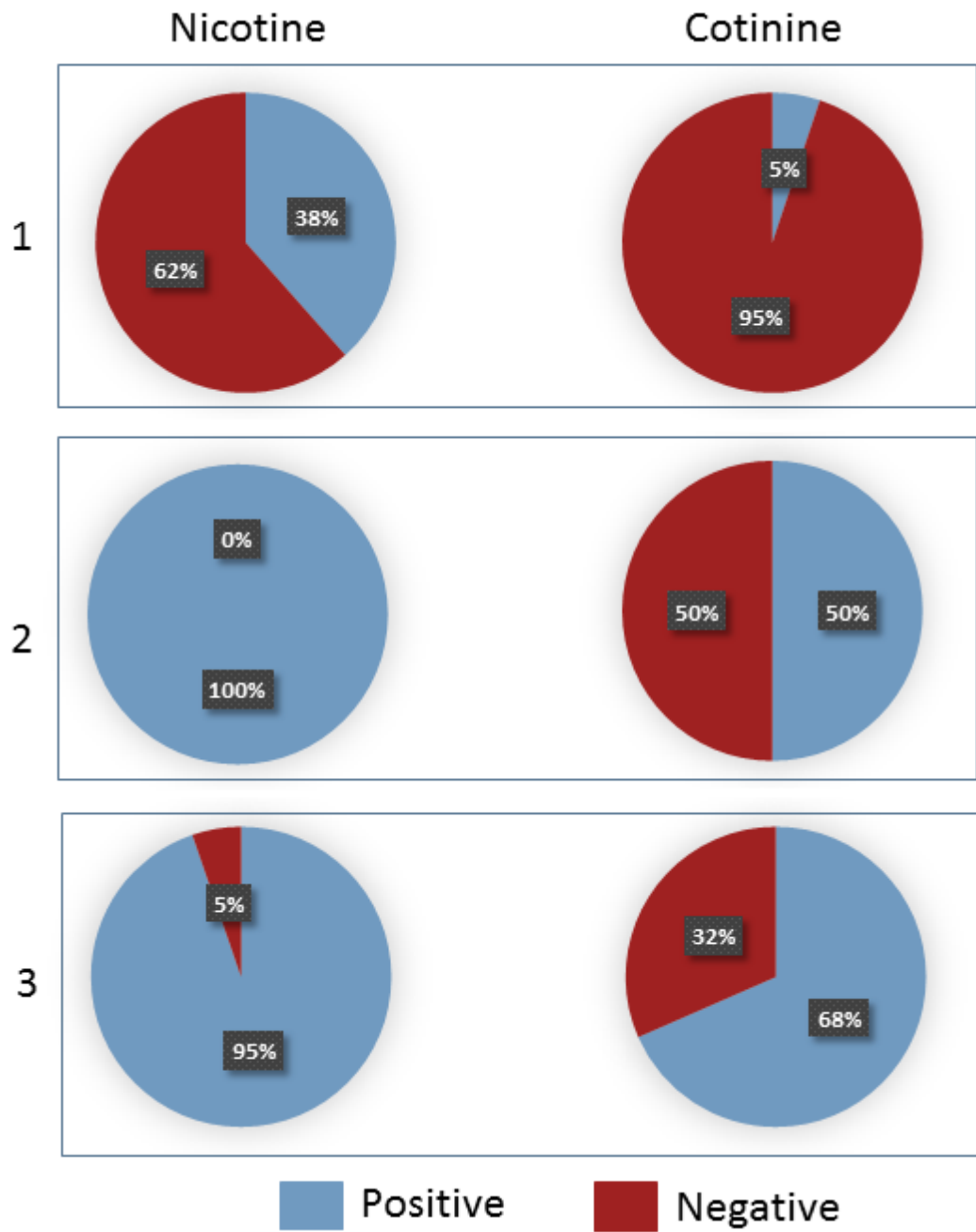
**Table 8-6 Cotinine results for exposed and non-exposed dogs**

	Smoking status		Totals
	Not exposed	Exposed	
<b>Test positive</b>	2	18	20
<b>Test negative</b>	38	8	46
<b>Totals</b>	40	26	66

**Table 8-7 Sensitivity, specificity, predictive values and likelihood ratios of nicotine and cotinine tests**

Assay	Sensitivity	Specificity	PPV	NPV	LR+	LR-
Nicotine	96.15	60.1	60.9	96	2.4	0.06
Cotinine	62.2	95	90	82.6	13.8	0.32

The following pie charts in Figure 8-7 show the proportion of positive and negative samples for nicotine and cotinine in each exposure group.



**Figure 8-7** Pie charts showing the proportion of dogs in each smoke exposure group that were positive or negative for nicotine and cotinine. (1) no exposure, (2) occasional exposure, smoking occurs only outdoor, (3) passive smoking, smoking occurs indoor.

## 8.8 Discussion

### 8.8.1 Total nicotine and total cotinine concentrations; comparison with concentrations reported in children's hair

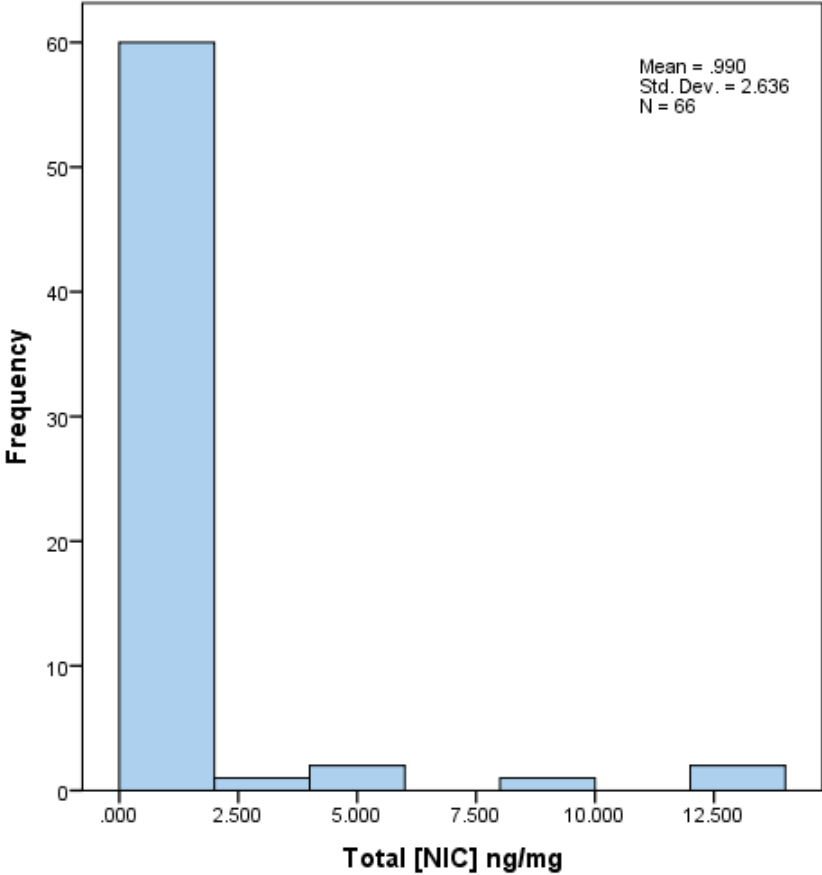
Nicotine in fur was measurable in most samples, while cotinine was often below the limit of quantification. Out of 66 fur samples, 41 samples were found to be positive with quantifiable amounts of nicotine and 19 samples only for cotinine. The mean concentration of nicotine in positive samples (1.6 ng/mg) was found to be approximately 5 fold higher than the mean concentration of cotinine positive samples (0.32 ng/mg). The nicotine concentrations ranged from 0.05 to 13.8 ng/mg and the cotinine concentration from 0.116 to 0.868 ng/mg. The distribution of concentrations is shown in Figure 8-8 (a) and (b). The concentrations detected for each analyte, as shown in Table 8-2, were compared to the concentrations detected in previous studies. Several studies have described hair nicotine concentrations for non-smoking children, or infants exposed to various amounts of tobacco smoke (239,241-244,252,267,270,271,286-296).

The mean fur nicotine concentration for dogs in our study was more than 2 times higher than the average of 0.8 ng/mg (interquartile range 0.27 to 2.24) measured in 1017 non-smoking children living in households with smokers in 31 countries (291,292). As our method did not include a washing step to remove nicotine adhering to the surface of the hair before hair analysis, it would be expected to provide higher concentrations that reflect internal and external doses. Pichini *et al* reported nicotine and cotinine concentrations from 24 infants who were classified into three groups similar to the classification in our study (Non-exposed (n=10), occasional exposure (n=7) and passive smoker (n=7)) (252). The mean nicotine concentrations were 1.3, 6.8 and 15.4 ng/mg for non-exposed, occasional exposed and passive smoker infants, respectively. Cotinine was not detected in all non-exposed infant's hair, and detected in one infant in the occasional exposed group with concentrations of 0.1 ng/mg, while in the passive smoker group, 5 out of 7 samples tested positive for cotinine with a mean concentration of 1.22 ng/mg ranging from 0.5 to 3.3 ng/mg. Despite the fact that Pichini's method included a wash step with 3 ml dichloromethane (x3), the reported mean concentrations for each group were found to be much higher than those found in our study, 0.16, 0.68 and 3.1 ng/mg for non-exposed, occasional exposed and heavily exposed.

It is believed that the lack of a standard method for estimated degree of exposure or differences in analytical methods could have caused this difference. For instance, Zahlsen *et al* found in a controlled ETS exposure environment that there is 4-10 fold variation in air nicotine levels for the same number of cigarettes smoked (318). The 'occasional exposure' in Pichini's study was defined as those infants with parents who smoked a maximum of 4-5 cigarettes per week even if smoking is taking place indoors, which would have been classified as heavily exposed in this study.

Moreover, the age of the infants in Pichini's study, ranges from 3 months to 36 months, suggesting that some infants may have been exposed to ETS in their neonatal environment. Interindividual variations including age, gender, race and other analytical aspects, such as presence or absence of a washing step, play roles in determining hair nicotine and cotinine concentrations. The anatomy of dog's fur and possibility of absorbing nicotine via licking the surface of their fur or contaminated objects limits further exploration of the basis for the differences in hair nicotine concentrations between our study and some studies on humans. Knottenbelt *et al* (297) reported concentrations of nicotine from 38 dogs exposed to different levels of ETS. The hair nicotine concentration (ranged from 0.11 to 11.31 ng/mg (median 0.57 ng/mg)) and is similar to that detected in our study.

(a)



(b)

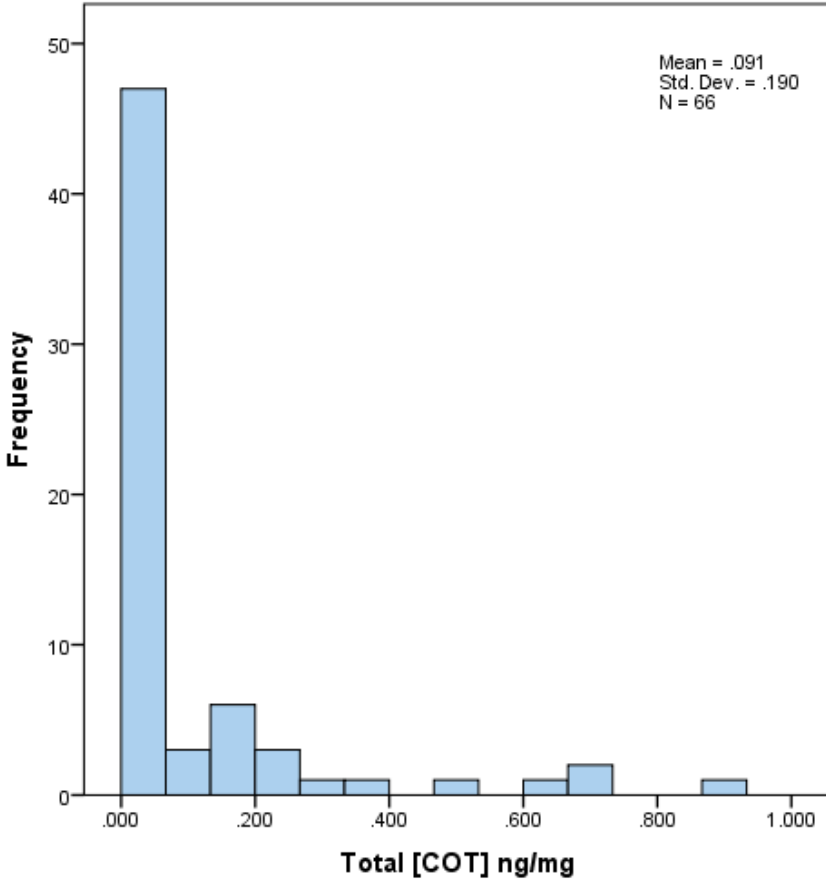


Figure 8-8 Histogram of (a) nicotine and (b) cotinine distributions in all 66 dog's fur

### **8.8.2 Differences between total nicotine and total cotinine concentrations in different smoke exposure groups**

As in this study, the majority of previous studies on ETS exposure used indirect exposure parameters by completing a questionnaire indicating their smoking habits, including location of smoking, number of cigarettes or number of smokers, rather than direct measurement of air nicotine or other chemicals as markers reflecting tobacco smoke exposure levels. Analysis of the fur specimens was blinded so that it was unknown which level of ETS each fur specimen was exposed to. Information regarding the ETS exposure was provided after the analysis was complete and is shown in Table 8-1.

The difference between the mean nicotine and mean cotinine concentrations was found to be significant between exposure groups. The mean nicotine concentration was found to be approximately six fold higher than the mean cotinine concentration in the 'no exposure' group, and about 3 fold higher in the occasional exposure group when smoking occurs only outdoor, and approximately 15 fold higher in the fur samples from dogs exposed to tobacco smoke indoor. Literature shows that the amount of cotinine in hair from smokers and non-smokers is at least 10 times less than nicotine. Descriptive statistics of nicotine and cotinine concentrations in each exposure group is shown in Table 8-8 and Table 8-9. The overlap in hair nicotine concentrations was observed between groups and may be due to the lack of standardised exposure estimation procedure or presence of unknown means of exposure.

The Kruskal-Wallis test was employed to test the null hypothesis of no difference in fur nicotine and cotinine concentrations between owner-reported exposure groups. The dog's fur concentrations of nicotine and cotinine appears to be closely associated with reported exposure to ETS. There was a statistically significant difference ( $P < 0.05$ ) in both fur nicotine and cotinine concentrations between all groups. Using the Mann-Whitney test, as post hoc test, it was found that the mean concentrations of nicotine and cotinine in non-exposed dogs were significantly different ( $P < 0.05$ ) from the mean concentrations of nicotine and cotinine in occasionally exposed dogs, and significantly different ( $P < 0.05$ ) from the mean value of nicotine and cotinine in passive smoker dogs. Furthermore, a statistical



difference existed between the mean concentrations of nicotine and cotinine in occasionally exposed dogs and in passive smoker dogs ( $P < 0.05$ ).

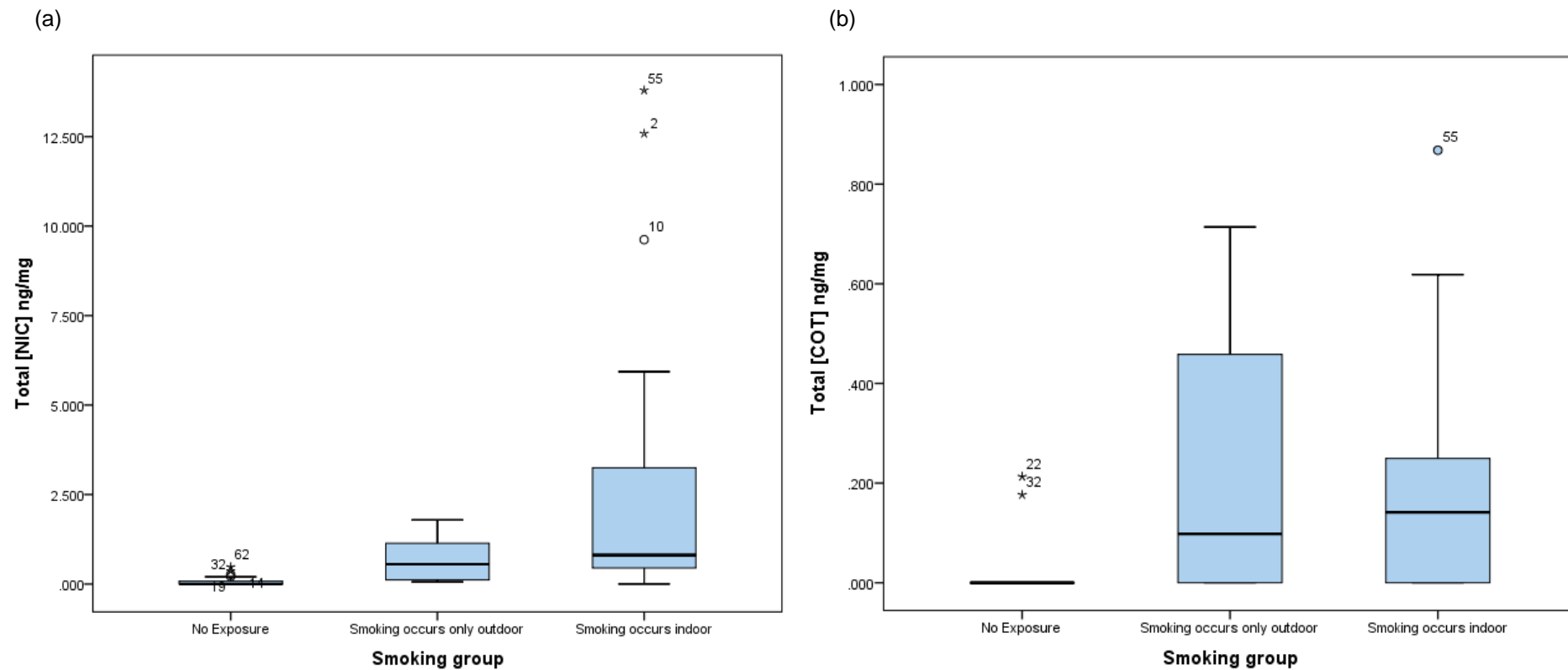
The above explained statistical significance suggests the potential utility of dogs' fur for monitoring ETS exposure. Boxplots of the distribution of hair nicotine and cotinine concentrations in the three exposure categories are shown in Figure 8-9.

**Table 8-8 Descriptive statistics of nicotine concentrations among different ETS exposure groups**

Smoking exposure group	Total [NIC] ng/mg				
	Count	Mean	Median	Minimum	Maximum
No Exposure	15	0.166	0.144	0.049	0.474
Smoking occurs only outdoor	8	0.683	0.553	0.062	1.791
Smoking occurs indoor	18	3.186	0.943	0.088	13.794

**Table 8-9 Descriptive statistics of cotinine concentrations among different ETS exposure groups**

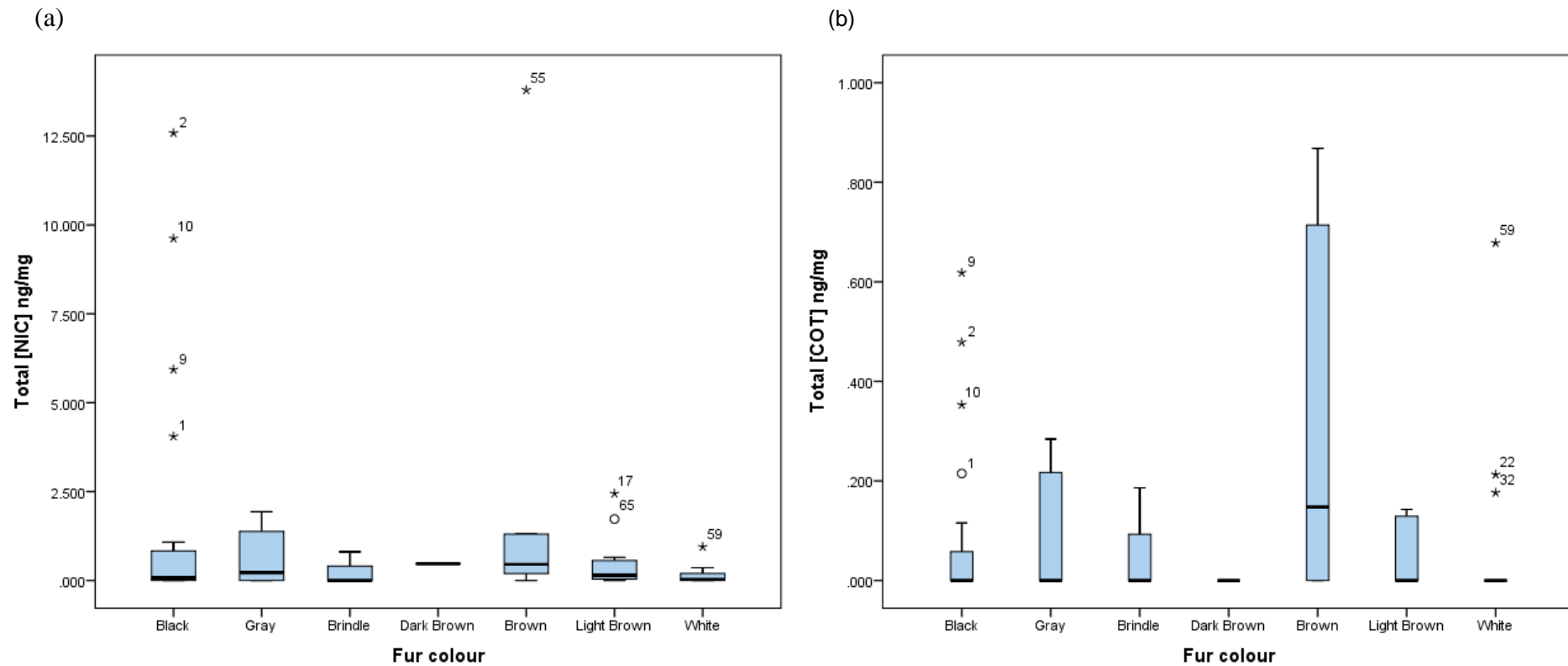
Smoking exposure group	Total [COT] ng/mg				
	Count	Mean	Median	Minimum	Maximum
No Exposure	2	0.195	0.195	0.176	0.213
Smoking occurs only outdoor	4	0.457	0.458	0.196	0.714
Smoking occurs indoor	13	0.293	0.186	0.116	0.868



**Figure 8-9** Boxplot diagrams representing the median and interquartile range of concentrations detected in fur samples exposed to different levels of tobacco smoke (a) nicotine (NIC) and (b) cotinine (COT). There was significant difference in the distribution of NIC and COT concentrations ( $p < 0.05$ ) detected between dogs with owners who do not smoke, smoke only outdoor and smoke indoor

### **8.8.3 Differences between total nicotine and total cotinine concentrations in different fur colours**

Hair colour could likely influence nicotine concentrations, since nicotine is bound to melanin and the type and amount of melanin in hair varies with hair colour. However, Zahlsen *et al* found that nicotine uptake did not differ due to hair colour or thickness (318). General comparison of nicotine and cotinine concentrations among all exposure groups using the Kruskal-Wallis test shows no significant difference between the seven colours. The same result was obtained after comparing concentrations within the same exposure group. It is noteworthy that the top four concentrations were detected in black fur. Due to the limited number of samples, for instance there was no white fur in the heavy exposed group, it was not possible to investigate the potential effect of colour bias. Boxplots of the distribution of hair nicotine and cotinine concentrations in the seven different fur colours are shown in Figure 8-10.



**Figure 8-10** Boxplot diagrams representing the median and interquartile range of concentrations detected in fur samples of different colours (a) nicotine (NIC) and (b) cotinine (COT). There was not significant difference in the distribution of NIC and COT concentrations ( $p=0.375$  for nicotine and  $p=0.485$  for cotinine) detected between different fur colour

#### 8.8.4 Differences between total nicotine and total cotinine concentrations at the two time points

Using the Mann-Whitney U test, there was no significant difference between the mean concentrations of total nicotine ( $p=0.442$ ) and total cotinine ( $p=0.875$ ) at the two time points for the twenty-five dogs returning for a second sample. Boxplots of the distribution of hair nicotine and cotinine concentrations at the two time points are shown in Figure 8-11. This result is not surprising as the majority ( $n=20$ ) did not report significant changes in their dog's exposure to ETS, whereas 5 dog owners only claimed a decrease in the exposure to different extent. Table 8-10 and Table 8-11 summarise the changes in concentrations of nicotine and cotinine and the owner-reported exposure levels at the two time points

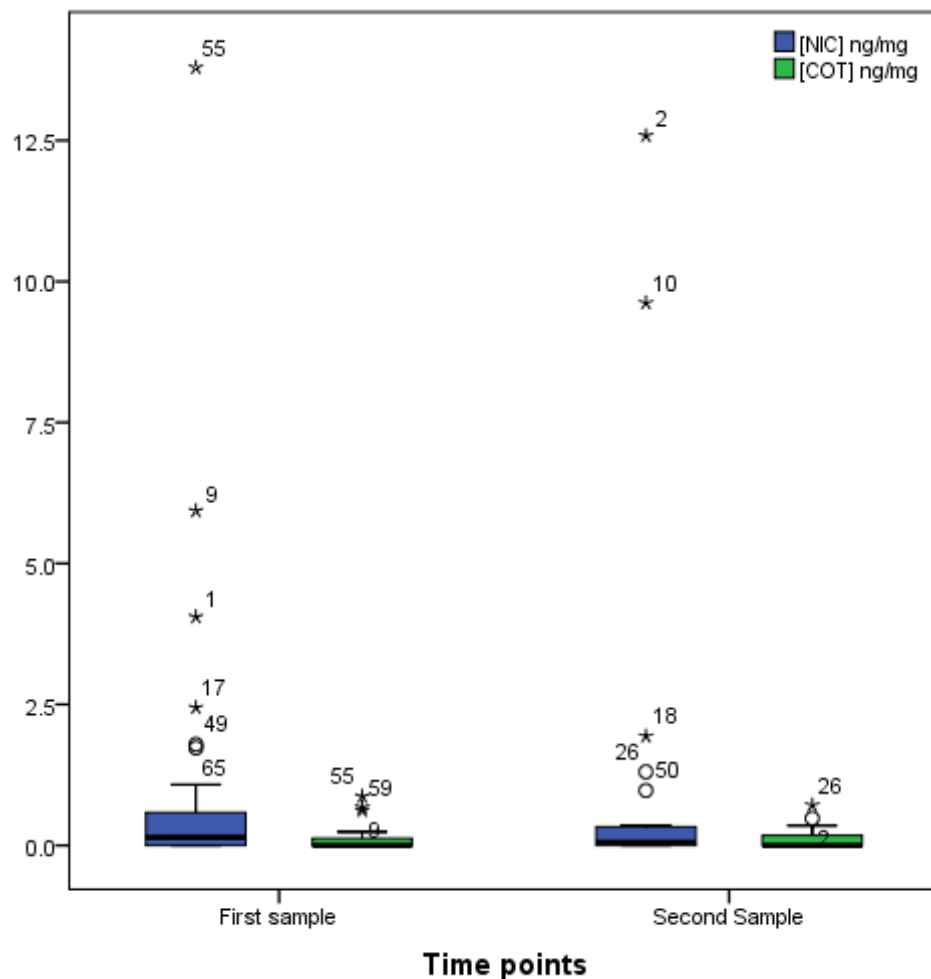


Figure 8-11 Boxplot diagrams representing the median and interquartile range of concentrations of nicotine and cotinine detected in fur samples at the two time points.

**Table 8-10 Summary of changes in nicotine concentrations and exposure status at the two time points.**

Sample ID	Time point 1		Time point 2		Exposure change	Nicotine change
	Smoking group	Total NIC (ng/mg)	Smoking group	Total NIC (ng/mg)		
1	3	4.052	3	12.585	=	↑
2	1	0.070	1	0.000	=	↓
3	1	0.000	1	0.000	=	=
4	3	0.440	3	0.328	=	↓
5	3	5.932	3	9.620	=	↑
6	1	0.000	1	0.000	=	=
7	2	0.143	1	0.360	↓	↑
8	1	0.000	1	0.000	=	=
9	3	2.443	3	1.935	=	↓
10	1	0.267	1	0.098	=	↓
11	3	0.357	1	0.201	↓↓	↓
12	1	0.000	1	0.000	=	=
13	3	0.454	2	1.306	↓	↑
14	1	0.000	1	0.000	=	=
15	1	0.000	1	0.000	=	=
16	3	1.078	1	0.211	↓↓	↓
17	1	0.000	1	0.000	=	=
18	1	0.077	1	0.148	=	↑
19	1	0.085	1	0.000	=	↓
20	3	0.808	1	0.088	↓↓	↓
21	1	0.000	1	0.058	=	↑
22	2	0.084	2	0.062	=	↓
23	1	0.049	1	0.000	=	↓
24	1	0.000	1	0.000	=	=
25	2	1.791	2	0.974	=	↓

The double arrow indicates that the level of exposure has changed significantly from heavy exposure to no exposure.

Despite the constant exposure at the two time points, higher nicotine concentrations were detected at the second time point for dogs 1, 5 and 18. This could be as a result of accumulation of nicotine over the time period with the constant exposure or may indicate that the data reported in the interview was incorrect. On the contrary, dogs 2, 4, 9, 10, 22, 23 and 25 have lower nicotine concentrations. Out of the five dogs, that were exposed to decreasing levels of ETS exposure, three have a lower nicotine concentration (dogs 11, 16 and 20) and two (dogs 7 and 13) have higher nicotine concentration. The owners of the three dogs with lower nicotine concentrations reported significant reduction in their dog's exposure to ETS and were moved from the heavy exposed dogs group to the non-exposed group.

**Table 8-11 Summary of changes in cotinine concentrations and exposure status at the two time points.**

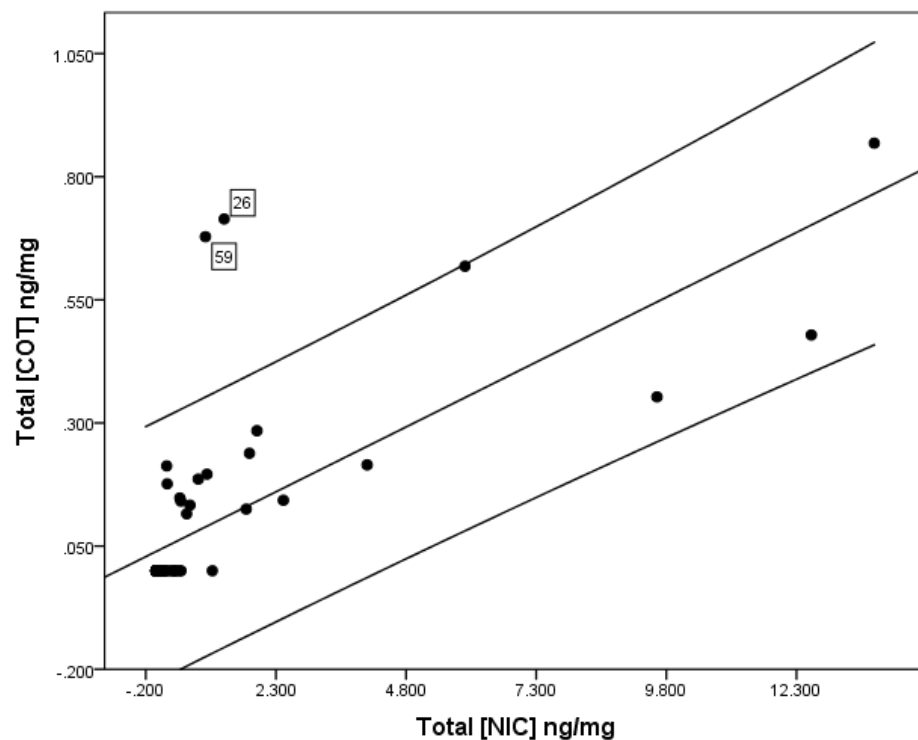
sample ID	Time point 1		Time point 1		Exposure change	COT change
	Smoking group	Total COT (ng/mg)	Smoking group	Total COT (ng/mg)		
1	3	0.215	3	0.479	=	↑
2	1	<LOQ	1	0.000	=	=
3	1	<LOQ	1	0.000	=	=
4	3	0.000	3	0.000	=	=
5	3	0.618	3	0.353	=	↓
6	1	0.000	1	0.000	=	=
7	2	0.000	1	0.000	↓	=
8	1	0.000	1	0.000	=	=
9	3	0.143	3	0.284	=	↑
10	1	0.000	1	0.000	=	=
11	3	0.000	1	0.213	↓↓	↑
12	1	0.000	1	0.000	=	=
13	3	0.148	2	0.714	↓	↑
14	1	0.000	1	0.000	=	=
15	1	0.000	1	0.000	=	=
16	3	0.000	1	0.176	↓↓	↑
17	1	0.000	1	0.000	=	=
18	1	0.000	1	0.000	=	=
19	1	0.000	1	0.000	=	=
20	3	0.186	1	0.000	↓↓	↓
21	1	0.000	1	0.000	=	=
22	2	0.000	2	0.000	=	=
23	1	0.000	1	0.000	=	=
24	1	0.000	1	0.000	=	=
25	2	0.238	2	0.196	=	↓

The double arrow indicates that the level of exposure has changed significantly from heavy exposure to no exposure.

Cotinine concentrations were not as sensitive as nicotine to the changes in ETS exposure. Increased cotinine concentrations were noticed for 3 dogs (11, 13 and 16) out of the 5 dogs exposed to lower levels of ETS. For dogs 4, 7, 9, 11, 14, 16, 17 and 21, different regions were reported for the second sample. However, no significant differences in the nicotine and cotinine concentrations were noticed.

### 8.8.5 Correlation studies of total nicotine and total cotinine concentrations

The Spearman's rho test was carried out to assess how well the relationship between nicotine and cotinine concentrations can be described. There was a strong positive correlation between the concentrations of nicotine and cotinine (Spearman  $\rho = 0.771$ ,  $p < 0.01$ ). The scatter plot diagrams for the correlation are shown in Figure 8-12. Natural logs of cotinine (ng/mg) and nicotine (ng/mg) provided to better visualise the correlation, however many data points were lost as it was not possible to take the natural log of 0, so the dogs with a cotinine of 0.000 were not represented.



**Figure 8-12** Scatter plot diagram showing the correlation between nicotine and cotinine concentrations in all 41 nicotine positive samples. Cases 26 (sample 13-B) and 59 (sample 34-A) have unusually high COT: NIC ratios. (Spearman's  $\rho = 0.733$ ,  $p < 0.01$ ).



### 8.8.6 Nicotine and cotinine concentrations in the methanolic wash and extract

The recovery of nicotine and cotinine from fur was assessed over a 2-hour sonication period and reached a plateau between 1-2 hours (see Figure 8-13 and Figure 8-14).

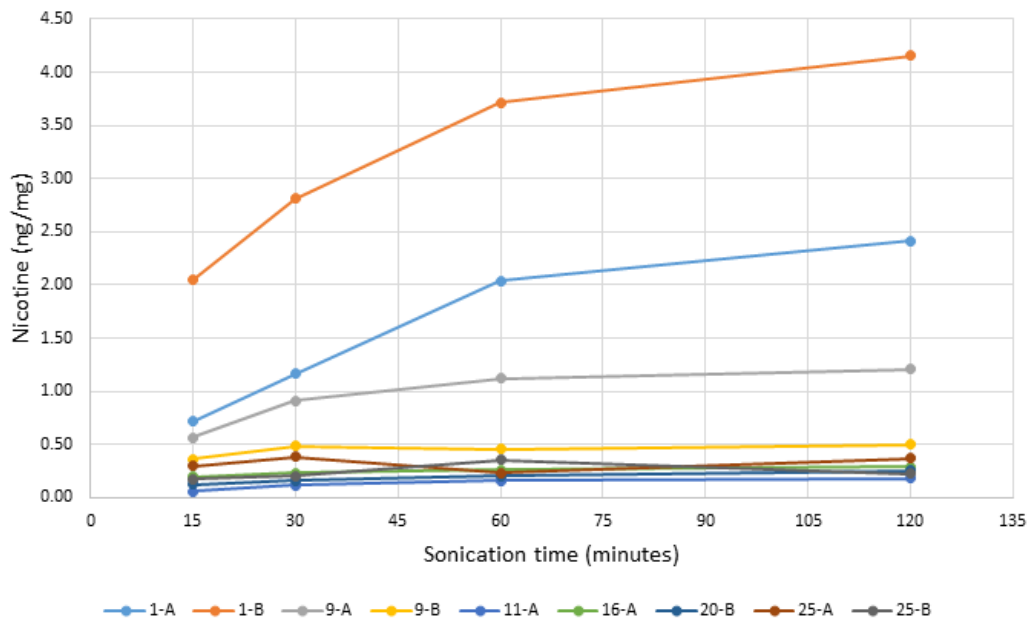


Figure 8-13 Dissolution of nicotine from dog's fur over 2 hours of sonication in methanol.

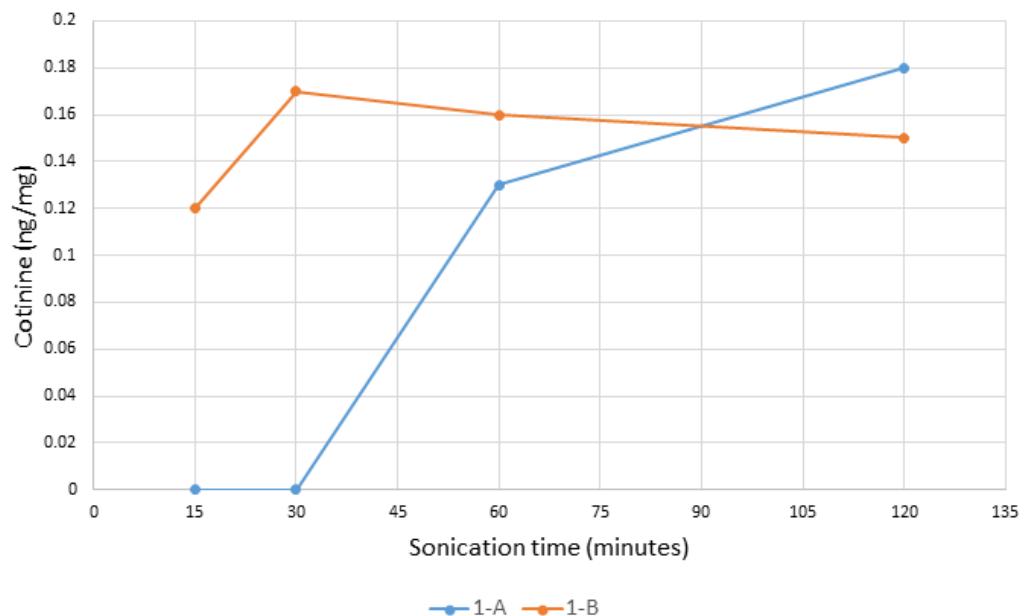


Figure 8-14 Dissolution of cotinine from dog's fur over 2 hours of sonication in methanol.

Nicotine was detected almost in all methanol washes for the 30 fur samples although only 15 were above the lower limit of quantitation (LLOQ) (0.05 ng/mg) at concentrations ranging from 0.05 to 5.92 ng/mg. The nicotine became undetectable after the methanol wash for four samples (10-B, 11-B, 20-B and 22-B). Cotinine was detected in about 43% of the methanol wash samples and above the LLOQ in seven samples at concentrations ranging from 0.1 to 0.31 ng/mg. After the wash, cotinine was undetectable in the extracts of five samples (9-B, 16-B, 20-A, 25-A and 25-B). The percentage of drug loss following the methanolic wash was measured by calculating the percentage of the concentration in the wash to the sum of the concentration of wash and after 120 minutes of incubation when detectable. Table 8-12 summarises the sum of wash and 120 min extract concentrations and the percentage of wash to the total concentration. Samples that had no detectable levels of nicotine and cotinine after the wash were excluded. The presence of cotinine in the methanol wash may indicate extraction of cotinine from inside the fur but could also be on the outer surface due to formation following tobacco smoking or via atmospheric oxidation of nicotine. The mean percentages for the amount of nicotine and cotinine that were removed from the outer fur surface were found to be approximately 52% and 55%, respectively (see Table 8-12). These results indicate that environmental passive exposure is the dominant contributor to the overall nicotine and cotinine found in hair both from non-smokers as it has been also suggested elsewhere (318,319).

**Table 8-12 Summary of sum of wash and 120 min extract concentrations and the percentage of wash to the total concentration**

	Nicotine		Cotinine	
	Total conc. (ng/mg)	%wash	Total conc. (ng/mg)	%wash
1-A	4.55	46.93	0.34	44.93
1-B	10.08	58.71	0.46	66.58
9-A	1.79	32.34		
9-B	1.48	66.44		
10-A	0.23	45.08		
11-A	0.30	37.83		
16-A	0.79	63.35		
16-B	0.14	64.99		
20-B	0.30	15.15		
25-A	0.94	60.86		
25-B	1.23	81.59		
Mean		52.12		55.75
Median		58.71		55.75
St. Dev.		18.77		15.31
%CV		36.02		27.46

The mean ratio of nicotine to cotinine in washes and in the 120-minute extract were calculated in 6 subjects (11 samples) and found to be highly variable (%RSD=88.4 and 89.47) as shown in Table 8-13. This finding is consistent with what has been reported in the literature. However, as two samples from each dog were tested, the within-subject ratios were found sometimes to be similar as shown with samples from dog number 25. This might be an indication of the ETS exposure pattern. This result suggests that the nicotine/cotinine ratio might be either subject-dependant or exposure-dependant. Furthermore, the nicotine/cotinine ratio has been reported not to be accurate at low ETS concentrations because background levels of respiratory suspended particles is believed to have an enormous influence on this ratio(237). Kintz *et al* reported a similar ratio for nicotine/cotinine ranging from 5-30 with a mean ratio of 10 in the hair of smokers (320).

Table 8-13 The values of nicotine and cotinine ratios in methanolic wash and extract

Sample ID	Nicotine/Cotinine ratio	
	Wash	Extract
1-A	14.16	1.30
1-B	19.26	26.97
9-A	34.54	17.79
9-B	5.82	7.86
10-A	16.14	6.02
11-A	24.19	6.91
11-B	0.96	3.25
20-A	6.11	1.73
20-B	2.65	4.83
25-A	3.35	7.04
25-B	5.25	9.88
Mean	12.04	8.51
Median	6.11	6.91
Standard Deviation	10.65	7.61
%RSD	88.45	89.47

### 8.8.7 Sensitivity, specificity, predictive values and likelihood ratios

The ideal test would be described as 100% sensitive and 100% specific; however, theoretically any test will possess a minimum error bound. Sensitivity and specificity of the nicotine test were found to be 96.15% and 60.9%, respectively. The cotinine test had better specificity with 95% and 62.2% for sensitivity. The two samples that were false positive in the non-smoking group and, therefore, resulted in a 5% decrease in cotinine specificity were taken from dogs that were previously classified as heavily exposed at the first time point.

The probability that the exposure to ETS is present was investigated using the positive predictive value (PPV) and was calculated to be 60.9% for nicotine and 90% for cotinine. In contrast, the probability that the exposure is not present when the test is negative were given by the negative predictable value (NPV) and found to be 96% and 82.6% for the nicotine and cotinine tests, respectively.

Likelihood ratios use the sensitivity and specificity of the test to determine whether a nicotine or cotinine test result usefully changes the probability that an ETS exposure exists. In general, a useful test provides a high positive likelihood

ratio and a small negative likelihood ratio.  $LR > 1$  indicates an increased probability that the ETS exposure is present, and a  $LR < 1$  indicates a decreased probability that the ETS exposure is present. The estimates presented here is based on the method reported by McGee(321). The nicotine test has a positive likelihood ratio of 2.4 which corresponds to an approximate 15% increase in probability of the exposure, and 0.06 negative likelihood ratio which corresponds to an approximate 45% decrease in probability. The cotinine test has a positive likelihood ratio of 13.8 which corresponds to an approximate 45% increase in probability, and 0.32 negative likelihood ratio which corresponds to an approximate 30% decrease in probability.

## 8.9 Conclusion

Nicotine and cotinine were quantified in 66 fur samples collected from 41 dogs exposed to different levels of ETS. The concentrations in dog's fur samples ranged from 0.05-13.7 ng/mg for nicotine, and 0.12-0.87 ng/mg for cotinine. These concentrations are similar to that reported in the literature after passive exposure to ETS in humans. The statistical analysis showed a positive association between home exposure to ETS and nicotine and cotinine concentrations. The mean value of nicotine and cotinine in non-exposed dogs was significantly different from the mean value of nicotine in occasionally or heavily exposed dogs. This strong association indicates that companion animals could be used to reflect the long-term ETS exposure in houses. However, complexity of estimation of ETS exposure and interindividual variability makes it difficult to estimate cumulative exposure accurately from single determinations. The sensitivity of nicotine for identifying heavily exposed dogs is high but has lower specificity, whereas cotinine has high specificity but lower sensitivity.

## Chapter 9 Conclusion and future Work

### 9.1 Cannabinoids in human hair project

#### 9.1.1 Method development

The work presented here has described the development of two methods for the analysis of cannabinoids in hair matrices. Firstly, a method for the analysis of cannabinoids (THC, CBN, CBD and 11-OH-THC) using LLE and standard GC-MS was developed and validated. The method was found suitable for analysis of authentic hair samples collected from known cannabis users. One of the frequently reported LLE disadvantages is that it results in waste of organic solvents. However, it was possible here to carry out a LLE with only 1.5 mL cyclohexane: EtOAc. The extraction recovery percentages were acceptable for all analytes. The produced extract was clean and the LOQ for THC was below the SoHT recommendation. The use of high recovery vials resulted in significant improvement in detection sensitivity. Secondly, a method for the analysis of the main metabolite THC-COOH in hair using SPE and 2D GC-MS was developed and validated. It was then used to analyse hair from known cannabis users and was shown to be suitable for the detection of THC-COOH in approximately 65% of samples. 2D GC-MS has been shown to be vital in the development of assays that require increased sensitivity over the use of standard GC-MS. The use of an ultra-inert column as the primary column has dramatically improved the detection sensitivity. However, further work may be required to improve the assay sensitivity as the LOQ was 5 times higher than that recommended by the SoHT. Two possible procedures for sample preparation were proposed and tested. The first procedure involved using a single hair sample, and started by carrying out LLE to extract THC, CBD, CBN and 11-OH-THC and followed by SPE of fraction 'B' for THC-COOH. The second procedure involved using two separate samples, LLE for sample (A) and SPE on sample (B). Both procedures were found to be acceptable. However, using a single hair sample for extraction of all cannabinoids was found to cause approximately 22.5% decrease in THC-COOH concentrations. The second procedure was employed for the two methods validation.

### 9.1.2 Application to authentic hair analysis

Validated methods were employed for the analysis of cannabinoids in 20 hair samples collected from known cannabis users. All samples were collected from cannabis user who reported use of amphetamine, simultaneously. It might be useful to compare the concentration obtained here with cannabinoids concentrations in hair samples from cannabis only users. The low detection rate of THC and high detection rate of CBD is not fully explained and needs further investigation. It is possible that THC is not stable in hair matrices as samples were stored for three months before analysis. It is also possible that the type of cannabis product available in the drug market in Saudi has a high CBD content. There was no significant statistical correlation or difference between the concentrations and weekly use score or among daily and non-daily users. A recent publication by Moosmann *et al* revealed that the detection of cannabinoids in hair does not prove cannabis consumption (322). After oral administration of 2.5 mg dronabinol (THC), three times a day over a 30-day period to two volunteers, there was no THC detected in their hair. This means that the external contamination, via smoke or handling of cannabis material, is the major route of THC incorporation into hair. Therefore, volunteers' exposure to cannabis smoke and material might be responsible for the hair concentrations not their use history. Studying the correlation between hair concentrations and cannabis smoke exposure would be an interesting subject for future work. A number of limitations has to be evidenced including; the limited number of analysed samples, lack of information on period of abstinence before sample collection, the lack of hair samples coming from abstinent users living with regular cannabis smokers and finally the lack of information on users' exposure to cannabis smoke.

### 9.1.3 Future work

Most of the work in the literature is targeting THC, CBD, CBN and the main metabolite THC-COOH, whereas a limited number of publications are available for analytes such as 11-OH-THC,  $\Delta^9$ -tetrahydrocannabinolic acid A (THCA-A) and 11-nor-D9-tetrahydrocannabinol-9-carboxylic acid glucuronide (THC-COOH-glu). The concentration ranges for these analytes and optimal analytical methods are still not fully established. 11-OH-THC is seldom targeted in hair samples to prove cannabis consumption. This is probably due to the assumption that 11-OH-THC is

present in much lower concentrations than the main metabolite THC-COOH. There are a limited number of studies where 11-OH-THC was included in the analytical method. 11-OH-THC was reported only in two papers. Shah *et al* quantified 11-OH-THC in 8 samples and the concentrations ranged from 0.61 to 3.1 pg/mg. Despite the fact that THC-COOH was included in the same method, it was detected only in one sample at 18.2 pg/mg. This might be an indication that despite the lower concentrations of 11-OH-THC, it has a higher detection rate than THC-COOH. Thieme *et al* published recently a method that involved an innovative derivatisation approach to enhance detectability of 11-OH-THC in hair. The 11-OH-THC was successfully quantified in one sample at 0.6 pg/mg. In the same sample, the THC-COOH was quantified at 1.5 pg/mg. THCA-A is the biosynthetic precursor for the main active ingredient in the cannabis plant, THC. Targeting the THCA-A is gaining increasing attention as a promising biomarker to distinguish illicit exposure to cannabis from the legal medicinal use. THC-COOH-glu is also a promising alternative to the main metabolite THC-COOH to prove consumption. Recently, Pichini *et al* reported a successful method for identification and quantitation of THC-COOH-glu in hair for the first time using ultra-high-pressure liquid chromatography-tandem mass spectrometry. The concentrations of THC-COOH in the same samples were found to be three times lower than that obtained for THC-COOH-glu.

Derivatisation is a very important part of the analytical method. It can dramatically improve detection sensitivity. However, the use of inappropriate derivation may lead to inaccurate quantitation results. Thieme *et al* reported a tremendous enhancement of mass spectrometric ionization efficiency for 11-OH-THC detection by formation of picolinic acid esters (198), and improvement in selectivity of THC-COOH detection in hair samples by selective methylation (163). Using 2-fluoro-1-methylpyridinium-p-toluenesulfonate (FMP-TS) in the presence of triethylamine (TEA), Fagehi *et al* developed a sensitive method for analysis of estrogens in plasma and serum (323). This derivatisation reagent has potential benefits for optimisation of detection sensitivity of the two metabolites 11-OH-THC and THC-COOH.

On the other other hand, a noteworthy issue with derivatisation of THC and CBD with PFPOH-PFPOH and TFAA-HFIP has been previously highlighted in the



literature. These two derivatising mixtures are commonly used to improve detection of THC-COOH by GC-MS in NCI mode. However, they have also been employed for the analysis of other cannabinoids including THC. Later publications have demonstrated that derivatisation with PFPOH-PFPOH (115,324) or TFAA- HFIP (165) is unsuitable for analysis of THC and CBD, as it resulted in identical retention time and mass spectra for THC and CBD, and hence incorrectly high concentrations of THC. The suitability of other perfluorinated anhydrides, such as HFBA, alone or coupled with perfluoroalcohols, such hexafluoropropanol (HFPOH), have not been investigated for analysis of THC and CBD yet. This combination HFBA-HFPOH, in particular, has been reported in at least one publication for analysis of THC in hair matrices (128).

## **9.2 Nicotine in dog's fur project**

### **9.2.1 Method development**

The first aim of this project involved the development and validation of a method for the analysis of nicotine and cotinine from dogs' fur. A simple methanolic extraction method was developed for the successful extraction of all analytes from the fur by 2 hrs sonication at 50 °C. Despite the good extraction recovery, it was not possible to concentrate the analytes after the initial extraction from fur. An LC system with tandem MS capability was employed for analysis. The conditions for multiple reactions monitoring (MRM) were optimized by autotuning during infusion of solutions of nicotine and cotinine.

### **9.2.2 Application to authentic hair analysis**

A total of 61 fur samples were analysed by the developed methods. Nicotine and cotinine concentrations detected in fur samples were significantly correlated with the exposure group. Nicotine was found to be more sensitive in measuring the ETS exposure changes, whereas cotinine was a more specific indicator of heavy exposure.

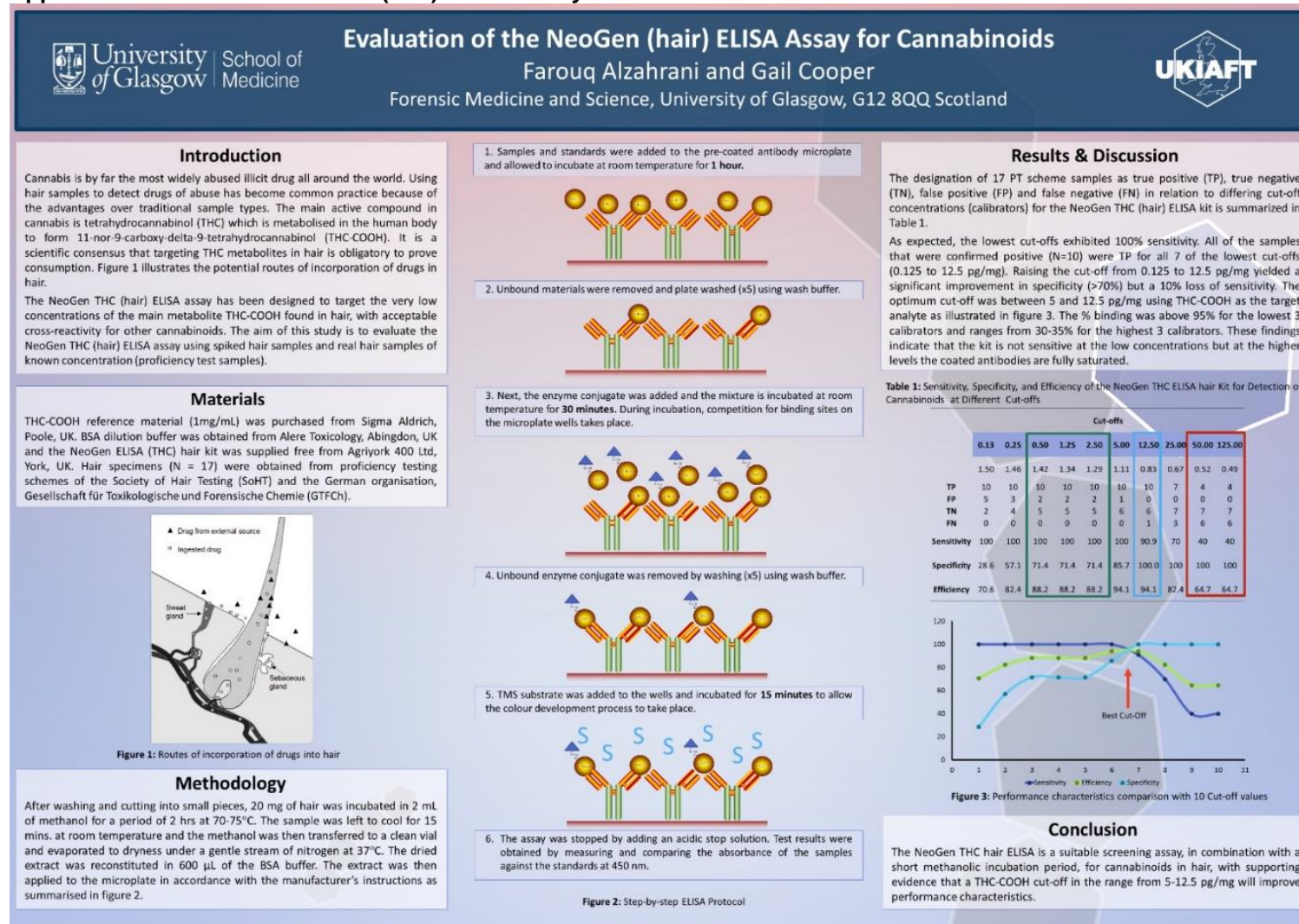
### **9.2.3 Future work**

Further research on the development of a more sensitive method for quantitation of nicotine and cotinine in hair with lower LOD and LLOQ would be of interest.

Both nicotine and cotinine are known to be present in ETS, therefore, including metabolites that originate exclusively from the human or animal body would help to distinguish the source of other compounds (external contamination or active use). Although methanolic extraction has proven to be a fast, cheap and effective method, the traditional liquid-liquid extraction or solid-phase extraction might result in a cleaner and concentrated extract, and hence better detection sensitivity. Another interesting research project would be comparing nicotine and cotinine concentrations in hair of children and pet dogs from the same house.

## Appendices

## Appendix I Evaluation of NeoGen (hair) ELISA assay for cannabinoids



# Appendix II Evaluation of two ELISA assays for the detection of cannabinoids in hair



## Evaluation of two ELISA assays for the detection of cannabinoids in hair

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### Introduction

Cannabis is by far the most widely abused illicit drug around the world. Using hair samples to detect drugs of abuse has become common practice because of the advantages over traditional sample types. Figure 1 illustrates the potential routes of incorporation of drugs in hair. The main active compound in cannabis is tetrahydrocannabinol (THC) which is metabolised in the human body to form 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH). The aim of this study was to evaluate the performance of two commercially available enzyme-linked immunosorbent assays for the detection of cannabinoids in hair.

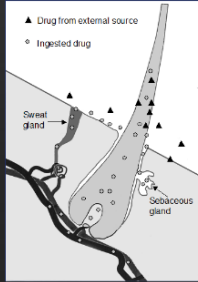


Figure 1: Routes of incorporation of drugs into hair

Table 1: Sensitivity, Specificity, Efficiency of NeoGen THC ELISA hair Kit for cannabinoids at different cut-offs

		Cut-offs									
		0.13	0.25	0.50	1.25	2.50	5.00	12.50	25.00	50.00	125.00
TP	10	10	10	10	10	10	10	10	7	4	4
FP	5	3	2	2	2	1	0	0	0	0	0
TN	2	4	5	5	5	6	6	7	7	7	7
FN	0	0	0	0	0	0	1	3	6	6	6
Sensitivity	100	100	100	100	100	100	90.9	70	40	40	40
Specificity	28.6	57.1	71.4	71.4	71.4	85.7	100.0	100	100	100	100
Efficiency	70.6	82.4	88.2	88.2	88.2	94.1	94.1	82.4	64.7	64.7	64.7

### Materials

THC and THC-COOH reference materials (1mg/mL) were purchased from Sigma Aldrich. The NeoGen ELISA (THC) hair kit was supplied by Agriyork 400 Ltd. Immunalysis ELISA Cannabinoids kit was supplied by Alere Toxicology, UK along with BSA dilution buffer, pre-incubation buffer, hair extraction buffer (HEB), neutralizing buffer (NEB) and phosphate buffered saline (PBS). Blank hair was collected from drug-free volunteers. Hair specimens (N = 17) were obtained from proficiency testing schemes of the Society of Hair Testing (SoHT) and the German organisation, Gesellschaft für Toxikologische und Forensische Chemie (GTFCh).

Table 2: Sensitivity, Specificity, and Efficiency of the NeoGen THC ELISA hair Kit for the detection of cannabinoids at 100 pg/mg cut-off.

	Analytes	
	THC	THC-COOH
Cut-off (pg/mg)	100	100
TP	10	8
FP	0	2
TN	7	7
FN	0	0
Sensitivity	100	80
Specificity	100	100
Efficiency	100	88.24

### Methodology

After washing and cutting into small pieces, 20 mg of hair was incubated in 2 mL of methanol for a period of 2 hrs at 70-75°C (for NeoGen kit) or sonicated in 1 mL of HEB for 2 hrs (for Immunalysis kit). The samples were then left to cool for 15 minutes at room temp. The methanol extract was transferred to a clean vial and evaporated to dryness with nitrogen at 37°C. The dried extract was reconstituted in 60 µL BSA buffer. 100µL was added to the microplate well. HEB extract was neutralized with 100 µL of neutralization buffer. 25µL was added to the microplate well after adding 25 µL pre-incubator buffer. Optimised ELISA protocols for both kits are summarised in figure 2.

The screening cut-off published in the latest SoHT guideline (2012) for cannabinoids (100 pg/mg) was used to determine if samples screened positive or negative for THC. However, due to the lack of a proposal for a screening cut-off for the main metabolite THC-COOH in the national and international guidelines, different cut-offs were tested for NeoGen kit to achieve the best performance characteristics.

### Conclusion

The NeoGen THC hair ELISA is a suitable screening assay, in combination with a short methanolic incubation period, for cannabinoids in hair, with supporting evidence that a THC-COOH cut-off in the range from 5-12.5 pg/mg will improve performance characteristics. Both kits performed well, with the Immunalysis kit better for targeting THC & the NeoGen kit better for targeting THC-COOH.

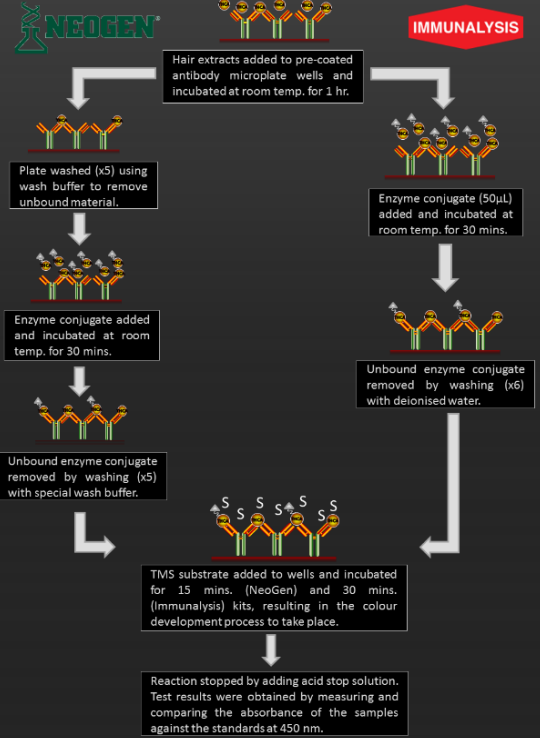


Figure 2: Step-by-step ELISA Protocols

### Results & Discussion

The designation of 17 PT scheme samples as true positive (TP), true negative (TN), false positive (FP) and false negative (FN) in relation to different cut-off concentrations (calibrators) for both immunoassay kits is summarised in Table 1 and 2.

For the NeoGen kit, the lowest cut-offs resulted in 100% sensitivity. All of the samples that were confirmed positive (N=10) were TP for all 7 of the lowest cut-offs (0.125 to 12.5 pg/mg). Raising the cut-off from 0.125 to 12.5 pg/mg yielded a significant improvement in specificity (>70%) but a 10% loss of sensitivity. The optimum cut-off was between 5 and 12.5 pg/mg using THC-COOH as the target analyte as illustrated in figure 3.

The % binding was above 95% for the lowest 3 calibrators and ranged from 30-35% for the highest 3 calibrators. These findings indicate that the kit is not sensitive at the lowest concentrations but at the higher concentrations the coated antibodies are fully saturated.

The Immunalysis kit targets THC but with good cross reactivity for the metabolite THC-COOH with 100 pg/mg utilised as the cut-off value. The sensitivity, specificity and efficiency for THC was better than that for THC-COOH. The presence of THC-COOH is an important indicator to differentiate between use and external contamination but is present at much lower concentrations than THC. An increased sensitivity to THC will identify many more presumptive positives hair samples but may also lead to more false positives.

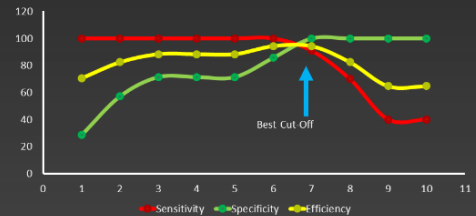
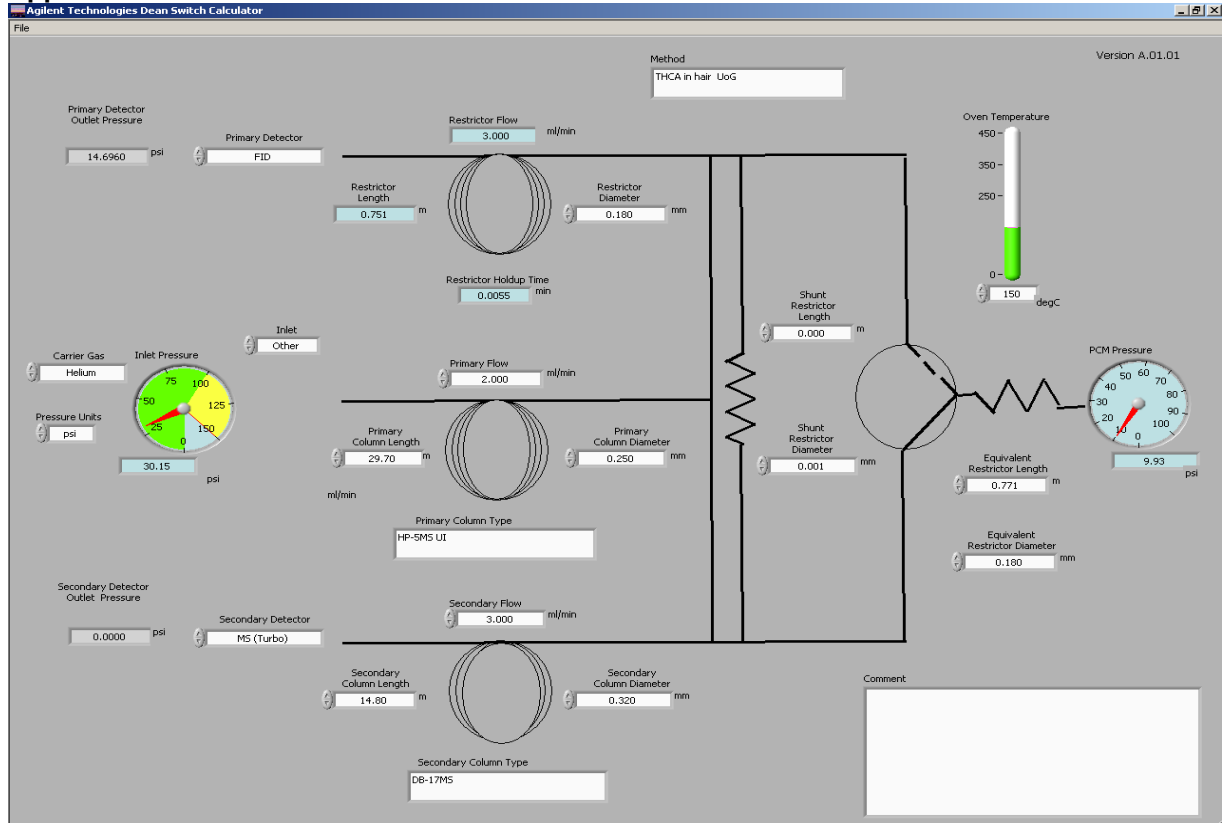


Figure 3: Performance characteristics comparison with 10 cut-off values

# Appendix III Screenshot of Dean's switch calculator software



Appendix IV Compounds (n=91) tested for interference study

Drug Group	Drug/Metabolite	Concentration (µg/ml)
Amphetamine type	Amphetamine	100
	Methamphetamine	
	MDMA	
	MDA	
	MDEA	
	PMA	
	PMMA	
Opiates	6MAM	0.1
	Morphine	5
	Codeine	
	Dihydrocodeine	
	Methadone	10
	Propoxyphene(Dextro-/Levo-)	
	Fentanyl	
	Norfentanyl	
	Oxycodone	
	Hydrocodone	
Opioid	Tramadol	10
	Dipipanone	
	Buprenorphine	
	Norbuprenorphine	
Stimulant	Cocaine	100
	Ecgonine methyl ester(EME)	
	Benzoylecgonine(BZE)	
	Cocaethylene	
Benzodiazepine	Chlordiazepoxide	10
	Diazepam	
	desmethyl-diazepam	
	Lorazepam	
	Oxazepam	
	Temazepam	
	Clonazepam	
	Nitrazepam	
	7-Aminoflunitrazepam	1
	Phenazepam	
	Etizolam	
	Pentobarbitone	100
	Phenobarbitone	
	Primidone	
Tricyclic antidepressant	Amitriptyline	10
	Clomipramine	
	Dosulepin	

	Doxepin	
	Imipramine	
	Desipramine	
SSRI	Citalopram	10
	Fluoxetine	
	Paroxetine	
	Sertraline	
Mixed-uptake inhibitor	Mirtazapine	10
	Venlafaxine	
Antipsychotic	Chlorpromazine	10
	Haloperidol	
	Risperidone	
Synthetic cannabinoid	CP47, 497	0.05
	JWH-250	
	JWH-073	
	JWH-018	
	JWH-200	
	JWH-018 5-OH pentyl	
	JWH-122 5-OH pentyl	
	JWH-210 5-OH pentyl	
	AB-FUBINACA	
	AM 2201 4-OH pentyl	
	AM 1248	
	HU-210	
Other	Caffeine	1000
	Procaine	
	Benzocaine	
	Paracetamol	
	Aspirin	100
	(-)-Cotinine	
	(-)-Nicotine	
	GHB	
	Ketamine	10
	Lidocaine	
	Trazodone	
	Diltiazem	
	Promethazine	
	Quetiapine	
	Cyclizine	
	Atenolol	
	Verapamil	
	Procyclidine	
	Zolpidem	
	Lamotrigine	
	Sildenafil	



Chlorpheniramine	
Phenytoin	
Diphenhydramine	
Carbamazepine	50

## Appendix V Ethical approval from Saudi MOH



Medical Research and Studies Department

المملكة العربية السعودية  
Kingdom of Saudi Arabia

مديرية الشؤون الصحية بمحافظة جدة  
Directorate of Health Affairs - Jeddah

Subject: Ethical Approval of Research Proposal.

Date: 15. Oct. 2015

To: University of Glasgow

We would Like to inform you that the following Research:

<b>Research Name:</b>	Farouq Faisal Hussain AL-Zahrani , DR. Ahmed AL - Asmaree
<b>Research Number:</b>	00579
<b>Approval Number:</b>	A00245
<b>Research Topic:</b>	Hair testing for drugs of abuse
<b>Approval Period:</b>	One year from the date of this letter

The IRP in the Directorate of health Affairs in Jeddah with registration Numbers (H-02-J-002) at the National Committee of Medical and bioethics has reviewed the research proposal and gave permission to start the research to the AL-AMAL Hospital in Jeddah.

Taking into consideration the following:

1. Follow the laws of the National Committee for medical and bioethics.
2. In the event of any change in the research plan you must obtain the approval of the research department.
3. Service not affected at the facilities concern.
4. Safeguard the rights and privacy of Persons subject to research.
5. The use of information for purposes of scientific research.
6. Submit a report on the progress of the study to the research department every six months.
7. Sampling in the research should be done under supervision of a specialist in the medical section.

Best Regard.

Head of Research Center - Jeddah

Dr / Mohammed Abdoul Raouf Tawfiq

الرقم: 302 / 26771 / 47 ج التاريخ: 2015 / 10 / 15 هـ الشفوعات: ١٥ / ١٥  
هاتف: ٠٢-٦٨٣١٣٧٧ / ٠٢-٦٩٧٠٠٠٦ فاكس: ٠٢-٦٦٢٢٩٦١ ص.ب: ١٢٤٩٣ جدة: ٢١١٧٦  
Tel.: 02-6831377. 02-6970006 Fax : 02-6622961 P.O. Box : 12493 Jeddah 21176  
موقع إلكتروني: www.mohj.gov.sa

## Appendix VI Ethical approval from University of Glasgow



5<sup>th</sup> November 2015

Dear Dr Gail Cooper, Dr Fiona Wylie and Farouq Alzahrani

### MVLS College Ethics Committee

**Project Title:** Validation of Analytical Methods for Cannabinoids in Hair Using Real Hair Samples – Proof of Cannabis Consumption

**Project No:** 200150013

The College Ethics Committee has reviewed your application and has agreed that there is no objection on ethical grounds to the proposed study. It is happy therefore to approve the project, subject to the following conditions:

- Project end date: August 2016
- The data should be held securely for a period of ten years after the completion of the research project, or for longer if specified by the research funder or sponsor, in accordance with the University's Code of Good Practice in Research: ([http://www.gla.ac.uk/media/media\\_227599\\_en.pdf](http://www.gla.ac.uk/media/media_227599_en.pdf))
- The research should be carried out only on the sites, and/or with the groups defined in the application.
- Any proposed changes in the protocol should be submitted for reassessment, except when it is necessary to change the protocol to eliminate hazard to the subjects or where the change involves only the administrative aspects of the project. The Ethics Committee should be informed of any such changes.
- You should submit a short end of study report to the Ethics Committee within 3 months of completion.

Yours sincerely

Prof. Andrew C. Rankin  
Deputy Chair, College Ethics Committee

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## Appendix VII Hair collection instructions

### Hair Collection Instructions (Living subjects)

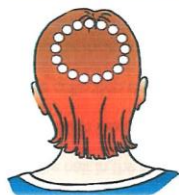
Please read through the checklist on the back page to ensure that all necessary steps have been completed. For further assistance regarding the hair collection procedure, please call +44 (0)141 330 4574.

1.



To avoid contaminating the hair sample, thoroughly clean a sharp pair of scissors with a sterile wipe. Allow the scissors to dry completely before proceeding.

2.



Identify the posterior vertex (back of the head) as shown by the circled zone. A lock of hair is required for the analysis (approximately the thickness of a pencil). Combing or brushing the hair prior to cutting may make separating out a bundle easier.

3.



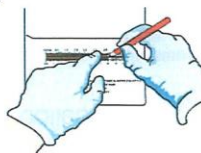
Once a suitable bundle of hair has been selected, separate it from the nearby hairs. Keep the bundle pinched securely in one hand whilst clearing the scalp area at the base of the bundle of any stray hairs.

4.



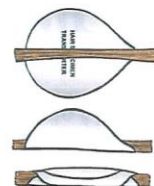
Using the clean scissors and keeping the sampled hair together, carefully cut the bundle as close as possible to the scalp. This may require a number of cuts. Make sure that the lock of hair is of pencil thickness in diameter. (This is the minimum required for analysis). If necessary, repeat steps 3 and 4 from several sites within the circled zone until a pencil thickness of hair has been collected.

5.



Keeping the lock of hair aligned, estimate the length of the hair collected using the scale on the collection envelope and record this on the toxicology request form. A description of the hair colour (including bleached or dyed) and appearance (e.g. straight, curly) should also be noted on the toxicology request form.

6.



Place the lock of hair along the length of the collection foil positioning the root end at the side marked "root end" and fold in the middle to secure. Fold the collection foil lengthways again to further secure. However, do not fold the sample back on itself. Place the foil in the collection envelope. If the sample is longer than the envelope then loop the hair back on itself but do not kink the hair by folding.

Complete the specimen identification details on the envelope and seal the envelope with the tamper proof seal. Complete the toxicology request form and enclose the sample and request form in the evidence bag. Deliver/send to Forensic Medicine and Science, University of Glasgow.

## Appendix VIII Consent form



University of Glasgow | College of Medical,  
Veterinary & Life Sciences

Centre Number:  
Project Number:  
Subject Identification Number for this trial:

## CONSENT FORM

**Title of Project:** Validation of Analytical Methods for Cannabinoids in Hair Using Real Hair Samples – Proof of Cannabis Consumption

Name of Researcher(s):

**Please initial box**

I confirm that I have read and understand the information sheet dated \_\_\_\_\_  
(version \_\_\_\_\_) for the above study and have had the opportunity to ask questions.

9

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my legal rights being affected.

7

I agree to take part in the above study.

--	--

Name of subject

Date \_\_\_\_\_

Signature

Name of Person taking consent  
(if different from researcher)

Date \_\_\_\_\_

Signature

Researcher

Date \_\_\_\_\_

Signature

(1 copy for subject; 1 copy for hospital)

## Appendix IX Interview form

**Donor Information***(To be filled in by sample collector)***Case number:** (will be filled in prior to sending collection kit)**Age of donor:****Sex of donor:** M/F ☐**Sample:** Hair ☐

1. Drug of abuse name(s) + the dose information:

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---



---

2. Please detail any prescription medication or any other medication taken by the donor in the past 12 months. If no medication has been taken please state "none".

---



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---

3. Has the hair been treated in any way (e.g. dying/bleaching) Yes/No  
(Please delete as applicable)**For Laboratory Use Only**

Date of receipt:

Hair colour (before treatment):

Hair length:

Segments used:

**Appendix X Calibration Model Calculations for the Weighting Factor****X-1 Introduction to curve weighting**

The assumptions for the non-weighted linear and quadratic regressions in bioanalytical LC-MS/MS assays are that the errors from the dependent variable ( $y$ , instrument response) are independent from each other (random), uncorrelated with the independent variable ( $x$ , concentration), and have equal variance ( $\sigma^2$ ) or standard deviation ( $\sigma$ ) across the different concentration levels (homoscedasticity). However, a quick review of any set of real life standard (STD) or quality control (QC) data from assay validations or sample analysis will reveal that the instrument response errors at different concentration levels are actually correlated with the independent variable (concentration) and have a larger variance ( $\sigma^2$ ) or standard deviation ( $\sigma$ ) at higher concentrations as long as the sample size at each concentration level is large enough to provide a rough estimate of variance. This is a clear indication that the instrument responses have heteroscedastic error. Due to this heteroscedastic error, the data at the high end of the calibration curve tend to dominate the calculation of the linear regression. This often results in excessive error at the bottom of the curve. One way to compensate for this error and to give a better fit of the experimental data to the calibration curve is to weight the data inversely with the concentration, a process called curve weighting. The weighting of calibration curves often will lower the overall error of the method and, thus, improve the quality of the analytical results. Most LC calibration curves that span several orders of magnitude show increasing error with increasing concentration, whereas the relative error (percent relative standard deviation, %RSD) is reasonably constant. Curve weighting should be evaluated whenever the relative error is fairly constant throughout the calibration curve.

**X-2 Evaluation of data**

A thorough evaluation of the appropriateness of curve weighting and selection of the weighting factor is best done at the end of method development or during method validation when a sufficiently large data set is available to calculate standard deviations at each calibrator concentration. Validation data was used for this purpose in this report. The first step is to prove that homoscedasticity was

not met for the analytical data and hence the need for a weighting factor (WF). Different ways are proposed for this purpose. The method that was chosen to test for heteroscedastic error was calculating the variance ( $\sigma^2$ ) or standard deviation ( $\sigma$ ) for all data calibration curve points as described in Gu *et al* paper (317). The next step was to determine the proper weighting factor for the data. Also different ways were proposed for this purpose. In this work, a weighting factor was selected using linearity indicators as described by Gu *et al*.

### **X-3      Heteroscedatic or homoscedatic error**

Tables X-1, X-2 and figures X-1, X-3 show the instrument responses of 11 calibration curves in five analytical runs for NIC and COT in dogs' fur LC-MS/MS assay validation. The standard deviations ( $\sigma$ , blue line) and variance ( $\sigma^2$ , red line) for instrument responses at each concentration level are shown in Figures X-2 and X-4. It was found that, from 0.01 to 10 ng/mg,  $\sigma$  increased in an approximately concentration-proportional manner, and  $\sigma^2$  increased in a much more than the concentration proportional manner. Error was exaggerated at each point so we can see the difference in error at the top and the bottom of the curve as shown in figures X-1 and X-3. This tells us that the absolute error is larger at the top of the curve than the bottom. If you follow the curve down, you can see that the points get closer together at lower concentrations. This behaviour tells us that the data are heteroscedastic and means that absolute error varies with sample concentration. Hence, there is a need for a weighting factor.



**Table X-1 Instrument responses of 11 calibrators (range 0.01-10 ng/mg) (n=5) for nicotine (NIC) in dogs' fur.**

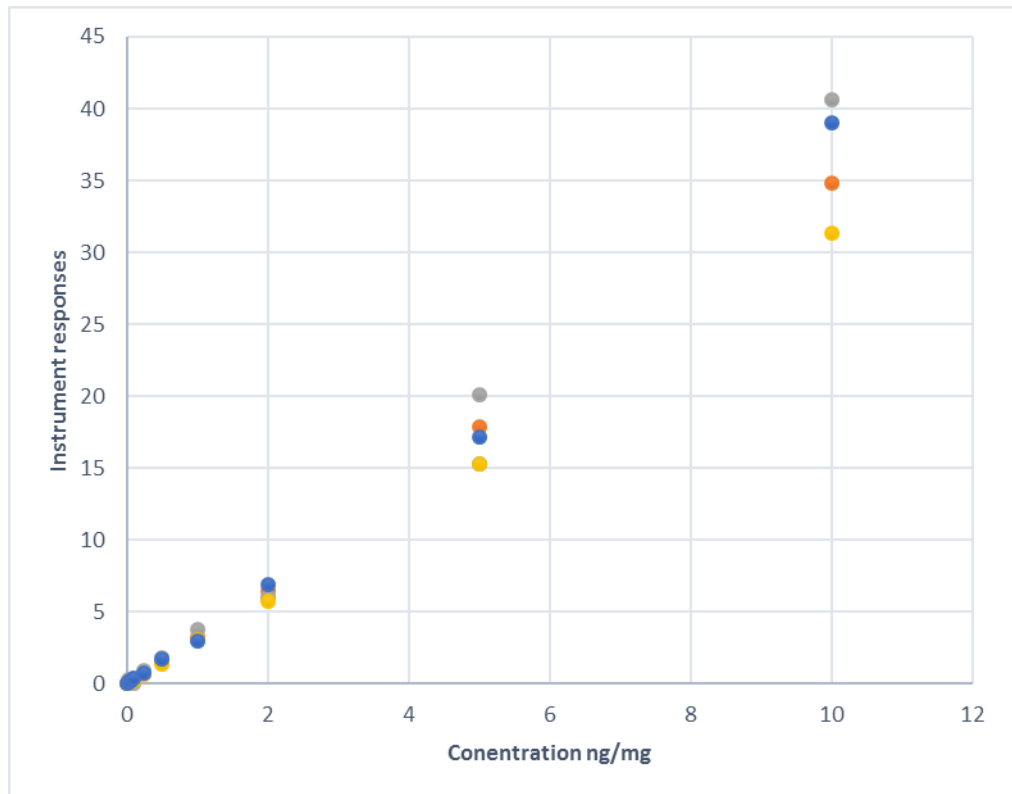
NICOTINE (PEAK ARE RATIO)						
	ng/mg	RUN1	RUN2	RUN3	RUN4	RUN5
CAL1	0.01	0.0424	0.0000	0.0000	0.0000	0.0539
CAL2	0.03	no data	no data	0.2952	0.0962	0.1239
CAL3	0.05	0.1654	0.1731	0.1900	0.1420	0.1822
CAL4	0.07	no data	no data	0.2641	0.1845	0.2330
CAL5	0.10	0.3550	0.2851	OL	0.2273	0.3806
CAL6	0.25	no data	no data	0.9163	0.6876	0.7915
CAL7	0.50	1.6066	1.5164	1.8296	1.3457	1.6999
CAL8	1.00	3.0594	3.2658	3.8056	3.0698	3.0032
CAL9	2.00	5.9038	6.4451	6.0472	5.7331	6.8801
CAL10	5.00	15.2727	17.8612	20.1482	15.2477	17.1810
CAL11	10.00	OL	34.8632	40.6195	31.3548	39.0060

OL stands for outlier

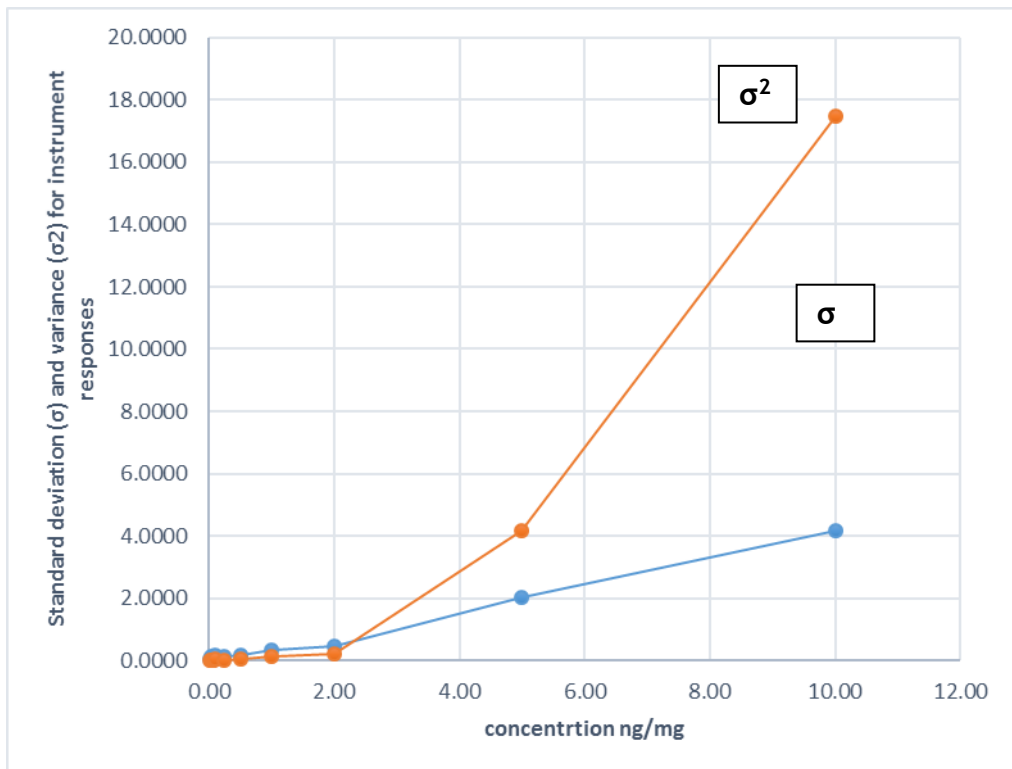
**Table X-2 Instrument responses of 11 STD curves in five analytical runs for COT in dog's fur LC-MS/MS assay validation (STD curve range: 0.01–10 ng/mL)**

COTININE (PEAK AREA RATIO)						
	ng/mg	RUN1	RUN2	RUN3	RUN4	RUN5
CAL1	0.01	0.0216	0.0165	0.0176	0.0149	0.0204
CAL2	0.03	no data	no data	0.0670	0.0427	0.0436
CAL3	0.05	0.0801	0.0758	0.0574	0.0756	0.0712
CAL4	0.07	no data	no data	0.0742	0.0938	0.0882
CAL5	0.10	0.1411	0.1258	0.1045	0.1251	0.1242
CAL6	0.25	no data	no data	0.3007	0.3463	0.2862
CAL7	0.50	0.7443	0.7263	0.6833	0.7336	0.6006
CAL8	1.00	1.3973	1.2404	1.1517	1.4406	1.0132
CAL9	2.00	2.7705	2.3902	3.8056	OL	2.2118
CAL10	5.00	7.3246	6.4112	5.9771	6.5557	5.3127
CAL11	10.00	OL	13.3071	11.6176	13.5272	11.1271

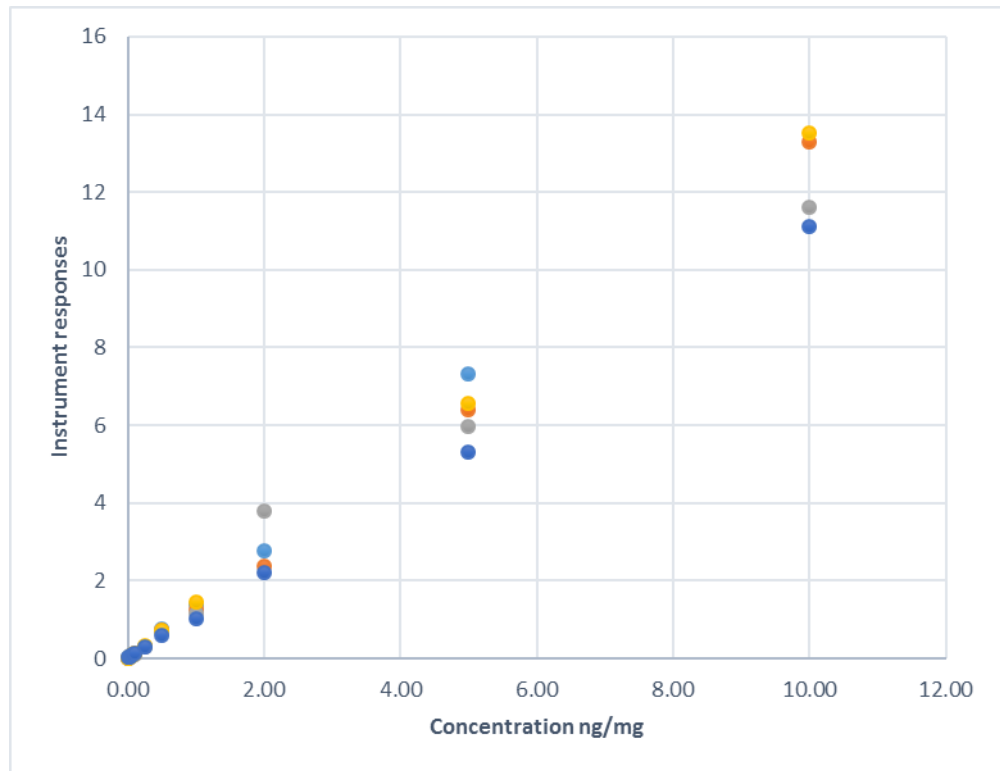
OL stands for outlier



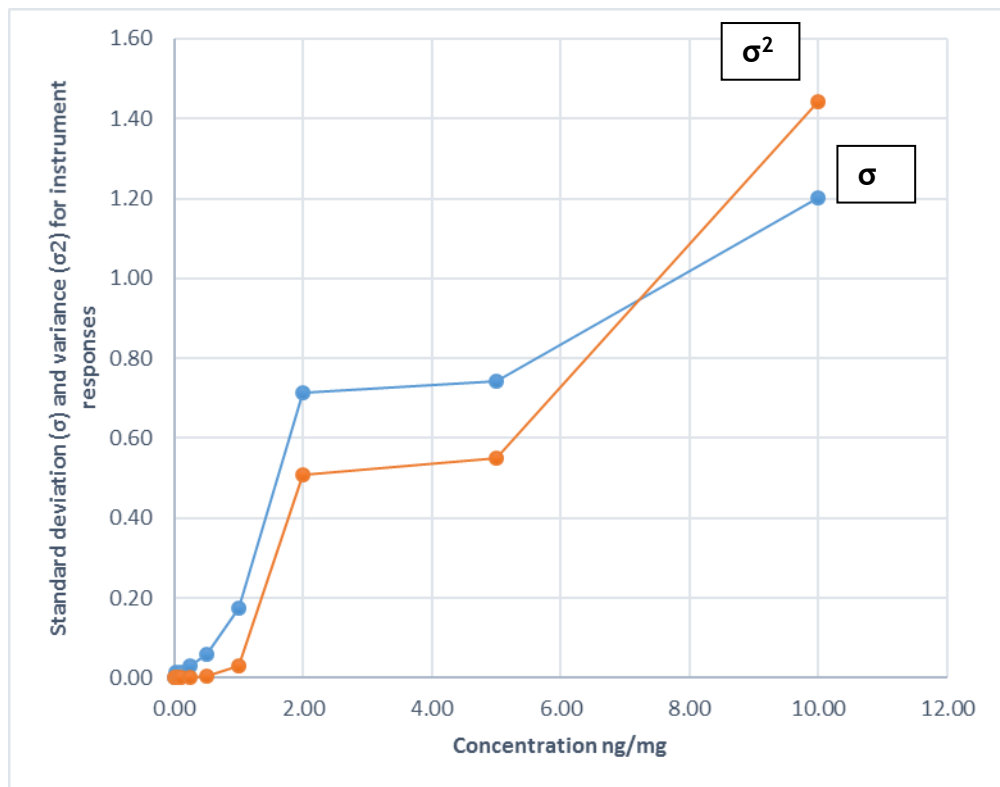
**Figure X-1 Instrument responses of 11 calibrators (range 0.01-10 ng/mg) (n=5) for nicotine (NIC) in dogs' fur.**



**Figure X-2 Standard deviation (σ) and variance (σ²) for Instrument responses of 11 calibrators (range 0.01-10 ng/mg) (n=5) for nicotine (NIC) in dogs' fur.**



**Figure X-3 Instrument responses of 11 calibrators (range 0.01-10 ng/mg) (n=5) for cotinine (COT) in dogs' fur.**



**Figure X-4 Standard deviation ( $\sigma$ ) and variance ( $\sigma^2$ ) for Instrument responses of 11 calibrators (range 0.01-10 ng/mg) (n=5) for cotinine (COT) in dogs' fur.**

## X-4 Selection of Weighting Factor Using Linearity Indicators

In some cases, the selection of a relationship with better linearity may not be obvious using the plots. An alternative, less empirical approach using three linearity indicators is proposed for the selection of weighting factor. The linearity indicators were defined as the relative standard deviation (RSD%) of  $\sigma/x^0$ , RSD% of  $\sigma/x^{0.5}$ , and  $\sigma/x^1$ , respectively. It is easy to see that, to justify the use of 1,  $1/x$  or  $1/x^2$  as the weighting factor, the RSD% of  $\sigma/x^0$ ,  $\sigma/x^{0.5}$ , or  $\sigma/x^1$  should be 0% as an exact linear relationship exists between  $\sigma$  and  $x^0$ ,  $\sigma$  and  $x^{0.5}$ , or  $\sigma$  and  $x$ , respectively, as shown in Table X-3. Therefore, in evaluating a real STD or QC data set, the best linear relationship can be assumed to be the one that gives the smallest linearity indicator. The linearity indicators were calculated for both NIC and COT assays. The results (Table X-3) confirmed the selection of  $1/X^2$  as the weighting factor for both assays. Please note that outliers were removed.

**Table X-3 Theoretical data for  $x$  and  $\sigma$  to justify the Selection of 1,  $1/x$ , or  $1/x^2$  as the Weighting Factor**

		0.01	0.03	0.05	0.07	0.10	0.25	0.50	1.00	2.00	5.00	10.00	$\sigma$	%RSD	WF
NIC	$\sigma$			0.02	0.04		0.11	0.18	0.33	0.46	2.04	4.18	1.47	160.07	
	$\sigma/x^{0.5}$			0.08	0.15		0.23	0.26	0.33	0.33	0.91	1.32	0.43	95.85	
	$\sigma/x^1$			0.37	0.57		0.46	0.37	0.33	0.23	0.41	0.42	0.10	25.16	$1/x^2$
COT	$\sigma$	0.00	0.01	0.01	0.01	0.01	0.03	0.06	0.18	0.71	0.74	1.20	0.42	154.23	
	$\sigma/x^{0.5}$	0.03	0.08	0.04	0.04	0.04	0.06	0.08	0.18	0.50	0.33	0.38	0.17	104.29	
	$\sigma/x^1$	0.28	0.46	0.17	0.14	0.13	0.13	0.12	0.18	0.36	0.15	0.12	0.11	55.84	$1/x^2$

**Appendix XI Oral and poster presentations in support of this thesis**

- Oral Presentation
  1. The Determination of THC-COOH in Hair using GCxGC-MS
    - Presented at 3<sup>rd</sup> annual Scottish Student Forensic Research Symposium (SSFRS), Glasgow, UK (Apr 2016)
  2. Detection Rates of  $\Delta^9$ -THC, CBD, CBN, 11-OH-THC and THC-COOH in Hair Samples from Middle Eastern Cannabis Users Using Validated Methods for GC-EI-MS and 2D GC-NCI-MS.
    - Accepted for oral presentation at and in proceedings of SOFT conference, Dallas, TX, USA, (October 2016).
- Poster Presentations
  1. Evaluation of NeoGen (hair) ELISA assay for cannabinoids
    - Presented at The United Kingdom and Ireland Association of Forensic Toxicologists (UKIAFT) meeting, Leicester, UK (August 2014).
  2. Evaluation of two ELISA assays for the detection of cannabinoids in hair
    - Presented at the 8<sup>th</sup> annual Saudi Students Conference (SSC), London, UK (Feb 2015).
  3. Determination of ETS Exposure in Pet Dogs Using Optimised Methanol Extraction of Fur Followed by ZIC-HILIC-tandem mass spectrometry Presented at and in proceedings of SOFT conference, Atlanta, GA, USA, (October 2015).
    - Presented at and in proceedings of SOFT conference, Atlanta, GA, USA. (October 2015).

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